REGULATION OF SPORULATION IN *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

During meiosis and spore formation in Saccharomyces cerevisae, changes in cellular polypeptides occur in a/α diploids but not in isogenic non-sporulating a/a strains placed under sporulation conditons. These were found by the technique of prelabelling growing cells with $^{35}SO_4^{2-}$ and resuspending them in sulphur-free sporulation medium. Under the conditions used, about 400 polypeptides were detected by two-dimensional gel electrophoresis, and 45 were altered during sporulation; of these, 21 changes were specific to a/α strains. These alterations were mainly due to the appearance of new polypeptides or to marked increases in the concentration of a few polypeptides that are produced during vegetative growth. They could have been due either to modifications to existing polypeptides present in growing cells, or to de novo synthesis of new products. The changes occurred at characteristic times during sporulation; while the majority took place early, within the first 6h in sporulation conditions, there were several changes characterising the later stages of sporulation.

Several sporulation-deficient mutants were isolated and two, 69.10C and XN129, were characterised genetically and biochemically. These two mutants were due to recessive mutations in genes specifically involved in sporulation, designated as spo50 for 69.10C and spo52 for XN129. They were blocked at two different stages : 69.10C at or near initiation and XN129 just after the onset of premeiotic DNA synthesis.

Protein extracts from 69.10C and XN129 were analysed by a technique similar to that used for the a/α sporulating diploid. Both mutant strains exhibited several sporulation-specific polypeptide changes. The pattern of changes, however, differed reflecting the stage at which each mutant was blocked in the morphological sequence. The polypeptide patterns also differentiated them from the non-sporulating a/a diploid. The presence of the a/α alleles allowed the appearance of several sporulation-specific polypeptides even in strain 69.10C which did not show any other recognisable sporulation specific events.

Cycloheximide, which inhibits protein synthesis on cytoplasmic ribosomes, added to sporulating cultures prior to extraction and analysis of polypeptides from the two mutants indicated that the appearances of a few sporulation specific polypeptides was resistant to the drug, suggesting that some of the changes are modifications which did not require de novo synthesis of new gene products or less likely they are proteins synthesised by the mitochondrial system.

To further the study of the pattern of gene expression during sporulation preliminary steps were taken towards the cloning of sporulation-specific genes. These indicated that it was possible to transform suitable diploid yeast strains using a vector YEp 13, that had been constructed from Escherichia coli plasmid pBR322, the LEU2 gene of Saccharomyces cerevisiae and a fragment of the 2μm circular DNA from yeast.

INTRODUCTION

INTRODUCTION

Sporulation in Saccharomyces cerevisiae furnishes a model of cell differentiation in a simple eukaryote. An understanding of its mechanism and control is not only of considerable academic interest, but could give ideas and information relevant to the study of differentiation at higher levels of cell development.

Yeast can exist stably in any state of ploidy up to hexaploid and conjugation can be easily manipulated. It presents many advantages for genetic studies, since it grows rapidly and it is easy to manipulate both biochemically and genetically. It is also now possible to carry out many in vitro genetic manipulation experiments. Its simplicity allows the use of many techniques applicable to bacteria such as replica-plating, micromanipulation and an array of biochemical procedures. In fact, apart from one or two bacterial species, Saccharomyces cerevisiae is one of the best characterised organisms in terms of biochemistry and genetics.

The study of sporulation in yeast is of fairly recent interest compared with that of bacterial sporulation as seen in Bacillus spp. Several groups of researchers have contributed to the basic understanding of the process, but a considerable amount has yet to be done to give a clearer, if not a complete understanding of sporogenesis in yeast. There are some similarities in sporulation of yeast and Bacillus spp. Both are initiated in response to a change in the nutritional levels and they exhibit an ordered sequence of morphological and biochemical changes. However sporulation in yeast involves meiosis and a high level of genetic recombination and these are events of significant

importance in the sexual reproduction of all eukaryotes. The ascus formed usually contains four ascospores which are the direct products of meiosis, while Bacillus spp. only produce a single spore in each mother cell.

LIFE CYCLE

Figure 1 illustrates the life cycle of the ascomycete Saccharomyces cerevisiae. Isolated spores germinated in appropriate nutritional conditions grow out to give haploid vegetative cells. Haploid cells consist of one of two mating types determined by two different alleles a and α at the mating type locus MAT. In strains carrying the ho allele (heterothallic strains), MATa and MATα are inherited as stable alleles of the mating type locus and produce clones of a and α cells. If they are separated, for example by dissection, they can undergo indefinite mitotic reproduction by mother cells budding off daughter cells. If haploid cells of mating type MATa are mixed with cells of mating type MATα, they each produce a hormone (a factor by a cells and α factor by α cells) that inhibits division and arrests cells of opposite mating type at a specific point in the cell cycle prior to the initiation of DNA synthesis (Bürcking-Throm et al., 1973). Arrested cells undergo a morphological alteration to an asymmetrically elongated form (Mackay & Manney, 1974) and cells of opposite mating type conjugate forming stable diploid zygotes which in turn reproduce by budding.

The presence of a dominant HO allele (which occurs less frequently in homothallic strains) changes the mating type of a growing bud to the complementary allele as often as every cell division according to specific rules of cell lineage (Hicks & Herskowitz, 1976; Strathern and Herskowitz, 1979). Once the new allele is expressed, nuclear fusion occurs which results in formation of a diploid heterozygous for the mating type locus. A 'cassette theory' has been

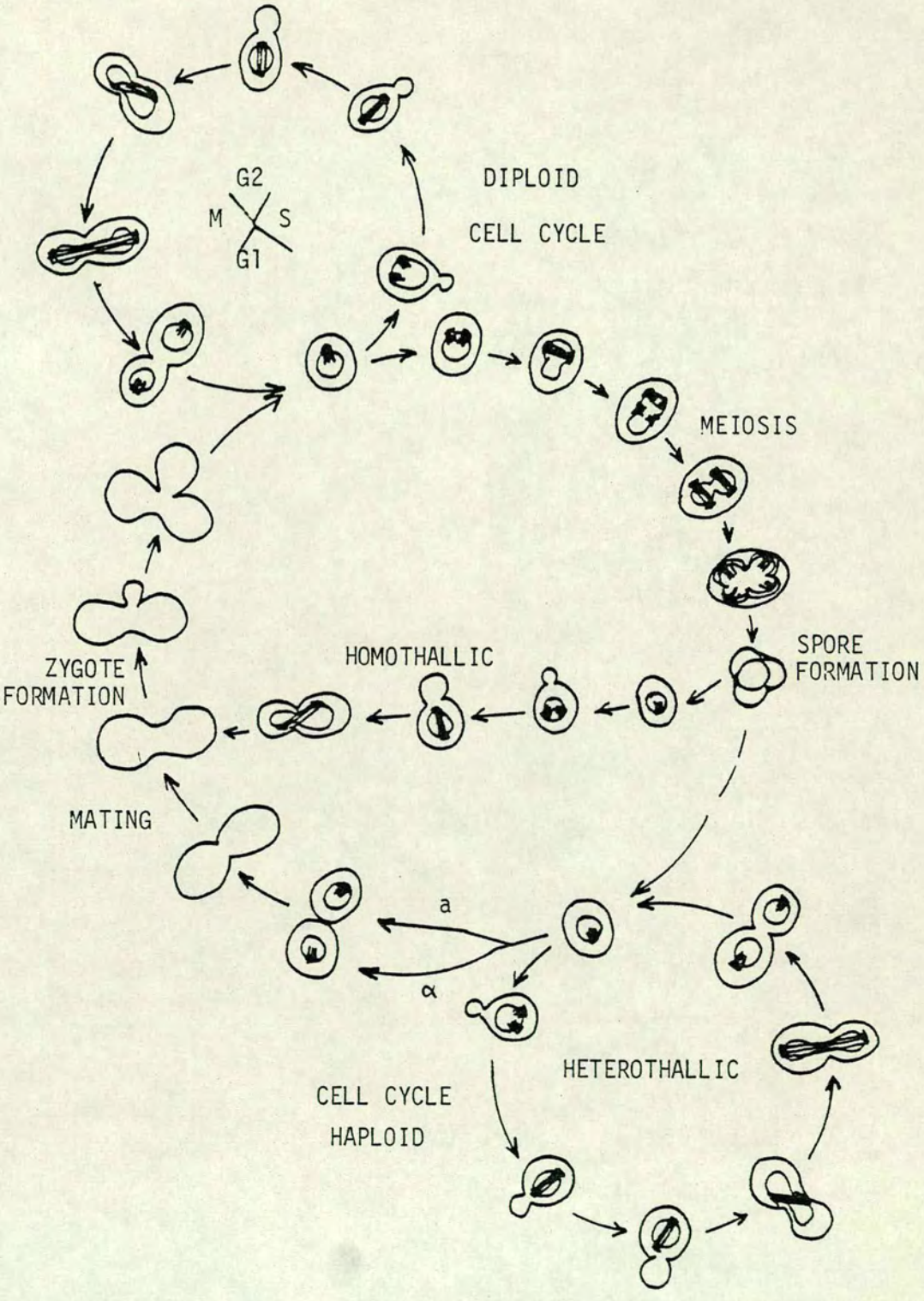


Figure 1. Life cycle of Saccharomyces cerevisiae

proposed for the interconversion of the mating type locus, (Hicks et al., 1977). Genetic studies revealed the existence of a silent copy of a information and another of α information and the HO gene activates this information by promoting the insertion of the copy into the mating-type locus. (Figure 2). The sites of silent α and a information are located at the HMLα and HMRa loci respectively. HML_{α} is required for switching MATa cells to MATα; HMRa is required for switching of MATα strains to MATa. HMRa was thus proposed to be silent MATa information and HMLα to be silent MATα*.

a/α diploids derived from both homothallic and heterothallic cell lines are unable to mate and do not produce or respond to mating pheromones. They can be induced to undergo meiosis and sporulation, a genetically and physiologically distinct phase in the life cycle of Saccharomyces cerevisiae. These phenomena are discussed in more detail in the subsequent sections.

*In subsequent discussion the mating-type alleles are designated as a (for MATa) and α (for MATα).

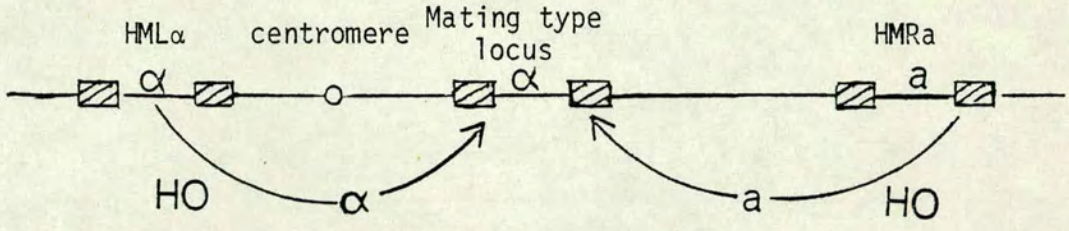


Figure 2: The 'cassette' model. Diagram of chromosome III (not drawn to physical or genetic scale) showing the mating type locus and HMRa and HMLα (which are closely linked to the mating type locus [Harashima & Oshima, 1976]). Boxes represent sequences involved in the recombination event mediated by the HO gene. Action of HO results in insertion of a and α cassettes into the mating type locus (Adapted from Hicks et al., 1977).

CYTOLOGY OF SPORULATION

The cytological changes that occur during sporulation are summarised in Figure 3. The early events are largely concerned with meiosis, and most of the obvious changes therefore involve the nucleus. Chromosome movement and morphology have not been resolved in Saccharomyces cerevisiae. Meiotic stages have been recognised by various staining techniques using light microscopy and these have shown changes and reorganisation of the nuclear structures. (Pontefract & Miller, 1962). Numerous electron microscopic studies have been attempted and the thin section work of Moens (1971) and Moens and Rapport (1971a and b) together with the freeze-etch studies of Hashimoto et al., (1960) have given the most acceptable reconstruction of cytological changes during meiosis and sporulation. From these an arbitrary classification of sporulation into various stages has been based to a large extent on the demonstration that spindle plaques attained characteristic configurations throughout meiosis. These stages can be summarised:

Stage I : On introduction into sporulation medium, cells have to enter the G1 phase of the cell division cycle before sporulation is initiated. At this stage, the single spindle plaque is small and indistinct, with very few microtubules and is located in the nuclear membrane. Towards the end of Stage I, synaptonemal complex elements become visible within the nucleus (Esposito of Esposito, 1974a).

Stage II : The spindle plaque duplicates and the two separate, eventually facing each other on either sides of the nucleus and they

are connected by a spindle of microtubules. There are also marked increases in number and size of lipid vesicles and endoplasmic reticulum membranes (Illingworth et al., 1973).

Stage III: The nucleus elongates as the spindle plaques move apart.

Stage IV : The plaques reach their maximum separation and each replicates for the second meiotic division. The nucleus assumes a four-lobed configuration, and prospore wall appears around each lobe (Moens, 1971; Moens & Rapport, 1971_a).

Stage V : All of the above events occur with the nuclear membrane intact. During stage V the lobed nucleus divides into four separate nuclei. Two spore-deliminating membranes derived from endoplasmic reticulum spread around each lobe; these join, and spore wall material becomes deposited between them (Illingworth et al., 1973; Lynn & Magee, 1970). The inner membrane becomes the plasmalemma of the spore and on the outer membrane the spore coat forms. Normally mature ascospores become visible under the light microscope 14 to 24h after resuspension of vegetative diploid cells into sporulation medium (Fowell, 1969).

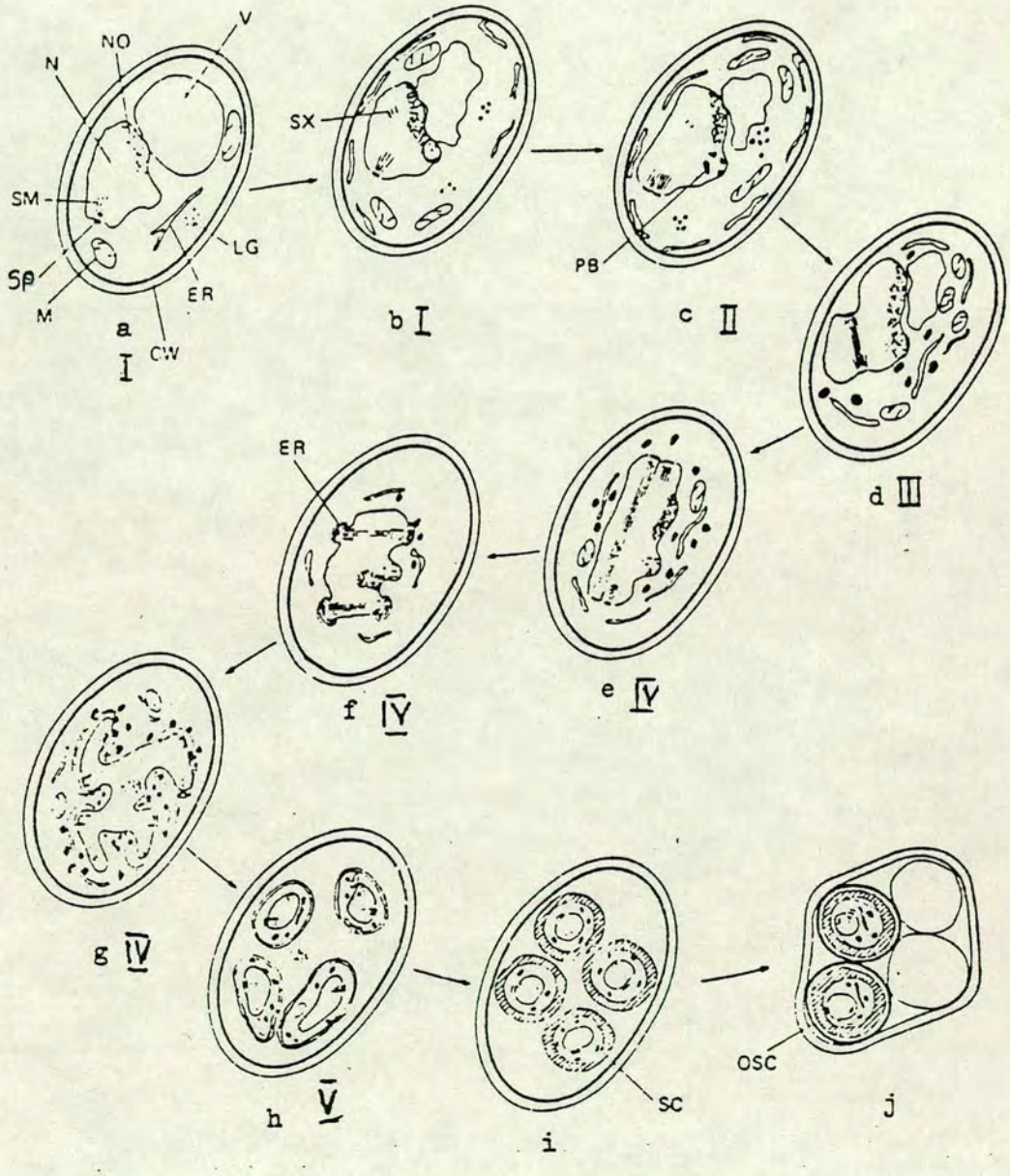


Figure 3 : The cytology of meiosis and spore formation in Saccharomyces cerevisiae. Redrawn after Esposito & Esposito (1974 a) and Fowell (1975). a,b.Stage I. c.Stage II. d.Stage III. e,f,g.Stage IV. h.Stage V. i,j.Spore maturation. The structures represented are: cell wall (CW); endoplasmic reticulum (ER); nucleus (N); nucleolus (NO); mitochondria (M); spindle plaque (SP); spindle microtubule (SM); lipid granule (LG); vacuole (V); synaptonemal complex (SX); poly-complex body (PB); spore coat (SC); outer spore coat (OSC).

BIOCHEMICAL EVENTS OF SPORULATION

Morphogenetic changes such as sporulation in Bacillus are usually accompanied by numerous, sequential, specific biochemical events. Studies of similar events in yeast are fairly recent and so far only a few biochemical markers have been attributed specifically to the process of sporulation. The change in nutritional conditions needed to induce sporulation enhances the difficulties encountered in trying to define these events, since starvation effects tend to mask sporulation events. To understand the process of sporulation and its regulation in molecular terms, at least some of the important events need to be identified and characterised as fully as possible, and this is one of the aims of the work done for this thesis.

All cell types, including the non-sporulating a/a and α/α diploids undergo a number of the metabolic responses that the a/α sporulating cells have on resuspension into sporulation medium. These a/a and α/α diploids are ideal as control strains in experiments to recognise sporulation-specific events since in these strains none of the biochemical and morphological changes characteristic of sporulating cells (i.e. premeiotic DNA synthesis, meiotic recombination; meiosis or ascospore formation) takes place under conditions in which a/α cells form ascospores.

Biochemical events essential but not specific to sporulation

Many of the changes that occur when cells are transferred into sporulation medium are changes that are brought about by the

the change in medium. Many of them may however be essential for the sporulation process.

RNA synthesis

The rates of synthesis of ribonucleic acids are similar in a/α, a/a and α/α non-sporulating diploids (Hopper et al., 1974). All species of RNAs are synthesised continuously with maximal synthesis fairly early in the process and another peak of activity roughly at the end of meiosis (Esposito et al., 1970). After this the RNA content declines steadily (Curiale et al., 1976; Esposito et al., 1969; Sogin et al., 1972). As during vegetative growth, most of the RNA transcribed is ribosomal RNA (rRNA), amounting to about 50% of the total rRNA at the end of sporulation (Sogin et al., 1972; Wejksnora & Haber, 1974). Processing of rRNA is however much slower compared with that in vegetative cells but it is not clear whether either the new synthesis or the altered rate of processing is essential for sporulation.

The a/α sporulating cells also accumulate an unmethylated 20S RNA in a 32S ribonucleoprotein particle that is resistant to ribonuclease digestion. The protein of the 32S particle is only synthesised under conditions inducing sporulation (Wejksnora & Haber, 1978). The relevance of this particle to sporulation is not known, as it is also found in methionine-starved vegetative cells (Wejksnora & Haber, 1974).

All isoacceptor species of transfer RNA (tRNA) are present during sporulation. There are some indications of difference in their amounts as compared to vegetative cells; this difference in isoaccepting species may play a role in the regulation of translation (Sogin et al.,

1972). Little is known on the proportion of messenger RNA (mRNA) transcribed during sporulation. Determination of poly (A)-containing RNA using Oligo (dT)-cellulose chromatography indicated that a significant amount is synthesised during the early part of sporulation (Curriale & Mills, 1975). Recently Mills; (Abstracts of 10th Int. Conf. Yeast Genetics & Molecular Biology, Louvain, 1980) has reported from hybridisation studies that there are some polyadenylated RNA species present in a/α sporulating cells that are absent from a/a diploids.

Protein synthesis

Continuous protein synthesis is an absolute requirement for spore formation since its inhibition at any time during the process prevented any further physiological and biochemical development except RNA synthesis, which was only slightly impaired (Magee & Hopper, 1974). The dependence on continuous protein synthesis persists until mature ascospores are observed (Esposito et al., 1969). Despite this requirement, the non-sporulating a/a and α/α diploids showed similar rates of synthesis under sporulation conditions (Hopper et al., 1974; Trew et al., 1979) as seen with RNA synthesis, the rate of protein synthesis is maximal fairly early in the process (Hopper et al., 1974; Petersen et al., 1979).

The extent to which protein synthesis is necessary for meiosis and sporulation is not known. In pulse-labelling experiments, some different proteins are synthesised so that polypeptide profiles seen by polyacrylamide gel electrophoresis (PAGE) are different when

compared with those from the vegetative cells (Hopper et al., 1974; Petersen et al., 1979). Undoubtedly, some of the new proteins are enzymes synthesised due to the release of catabolite repression, some of them may be directly or indirectly relevant to sporulation events. Electron microscopy has indicated the formation of structures such as meiotic spindle plaque and synaptonemal complexes which are absent in non-sporulating cells (Moens & Rapport, 1971a). It has also been shown that ascospores contain an immunologically distinct antigen that is absent from vegetative cells, and this has been tentatively identified as containing protein, (Snider & Miller, 1966). Despite all these findings attempts to identify proteins synthesised during sporulation that are specific to the process have yielded negative results (Hopper et al., 1974; Petersen et al., 1979; Trew, et al., 1979).

Requirement for derepression of some mitochondrial functions

Functional mitochondria are essential for sporulation. Petite strains, deficient in respiratory activity due to mutation in either the nuclear or cytoplasmic genome are unable to sporulate (Sherman, 1963). Inhibition of the development of the TCA cycle system also prevents the onset of sporulation, (Kuenzi et al., 1974; Miller & Halpern, 1956). This requirement is similar to that in sporulating Bacillus, where mutational loss of some TCA cycle enzymes or cytochromes leads to a block in the early stage of sporulation (Fortnagel & Freese, 1968; Taber et al., 1972). It was proposed that these mutants are unable to maintain the ATP level required for sporulation, as indicated by the observation that a malate

dehydrogenase mutant sporulated normally when supplied with oxaloacetate (Onne & Rutberg, 1976). Thus it seems that yeast may also need a certain level of ATP for successful sporulation.

Using erythromycin-sensitive strains Puglisi & Zennaroe (1971) demonstrated that sporulation was inhibited when the strains were treated with erythromycin, an inhibitor of mitodrondrial protein synthesis. However Kuenzi et al., (1974) were able to show that sporulation can occur in the absence of a significant amount of mitochondrial DNA replication and mitochrondrial protein synthesis provided the cells were pregrown in medium which allowed adaptation to oxidative metabolism. This was attained by controlled treatment of exponentially growing cells from acetate medium with ethidium bromide. The sporulation frequency was only slightly affected but the quality was rather poor, since mainly two spored asci were produced. When germinated, the spores have a similar phenotype to that of petite mutants, i.e. they were unable to utilise non-fermentable carbon sources.

The indirect effect of mitochondrial protein synthesis on sporulation was further demonstrated by studies of certain mit$^-$ mutants (Pratje et. al., 1979). These mutants retained a functional mitochondrial protein synthesis system, but produced defective cytochrome b and cytochrome c oxidase activities due to lesions in the mitochondrial genome. Most mit$^-$ mutants were unable to sporulate although about a third sporulated with decreased efficiently.

In summary, some but not all mitochondrial functions are

necessary for successful sporulation, and these functions depend on the existence of a mitachondrial system for protein synthesis. However, provided the cell is mitochondrially competent just prior to its resuspension in sporulation medium, no further mitochondrial synthesis appears to be necessary.

Utilisation of acetate

Acetate is utilised via the glyoxylate cycle during sporulation (Croes, 1967). Cells preadapted to oxidative metabolism consumed acetate immediately after transfer into sporulation medium (Esposito et al., 1969). The level of isocitrate lyase, one of the glyoxylate cycle enzymes was high at the onset of sporulation (Betz & Weiser, 1976). Croes (1967) proposed that sporulation may be triggered by an insufficiency of this cycle. In his view, the biosynthetic and energetic demands of the sporulating cells, in the absence of an adequate nitrogen source are too great to be satisfied by the output of the cycle. There is little direct evidence to support this hypothesis.

Studies using [^{14}C] acetate indicated that it is an important source of metabolites for sporulation. Most is converted to carbohydrates which accounted for about two-thirds of the total increase in mass during sporulation (Kane & Roth, 1974). These included storage materials, mainly trehalose and glycogen, and structural carbohydrate such as glucan and mannan. A fraction of it is also incorporated into lipid materials. (Henry & Halvorson, 1973; Illingworth et al., 1973). On completion of sporulation 62% of the

labelled acetate had been respired as carbon dioxide 22% was in the soluble pool and 16% had been incorporated into macromolecules (Esposito et al., 1969).

Biochemical events specific to sporulation

As indicated earlier, the number of known specific events accompanying the transformation of the vegetative cell into an ascus is surprisingly low. The cell takes full advantage of the other events that have to take place due to the reduction of the essential nutrients in the sporulation medium. The few events however, define clearly the requirements and physiological state of the cell undergoing meiosis and spore formation.

DNA synthesis and recombination

Initiation of meiotic DNA synthesis is the first detectable indication of the beginning of the sporulation process (Croes, 1966; Hopper et al., 1974; Roth & Lusnak, 1970; Simchen et al., 1972). Under normal conditions DNA synthesis begins after about four h of resuspension and is completed within the next six h (Esposito & Esposito, 1974a; Hopper et al., 1974; Simchen et al., 1972). The long duration, compared with mitotic DNA synthesis, is not due to a decrease in the rate of synthesis, but by a reduction in the number of initiation points per genome (Callan, 1972; Williamson et al., 1980). Differences in the rate and pattern of synthesis suggest that unique or modified gene products distinct from those of the vegetative growth may be required during meiosis. The mechanism of synthesis

is however very similar, as shown by the ability of cells that have started premeiotic DNA synthesis to revert to the mitotic cycle when provided with rich medium, utilising the DNA already synthesised (Simchen et al., 1972). At least part of the control of synthesis is also shared by the two cycles. Mitotic cell division cycle mutants (cdc mutants) with defects in initiation of replication or of DNA synthesis showed similar defects during meiosis (Hartwell, 1974; Simchem, 1974).

Meiotic DNA synthesis is accompanied by a high level of genetic recombination, detected shortly after the onset of DNA synthesis (Jacobson et al., 1975; Roth, 1973; Sherman & Roman, 1963). Recombination occurs at a very low level during vegetative growth (Hartwell, 1974). This highlights what may be one of the prime functions of meiosis, to provide variation in the population.

All sporulation mutants that have been shown unable to undergo premeiotic DNA synthesis also fail to exhibit intragenic and intergenic recombination (Esposito et al., 1975; Haber et al., 1975; Roth, 1973). Esposito & Esposito (1978) suggested that these two events are coordinately controlled. However, commit ment to recombination is not sufficient to commit the cell to meiotic divisions; this was shown by genetic analysis of cells which were returned to mitosis. They became committed to a meiotic frequency of recombination before they become committed to meiotic segregation (Esposito & Esposito, 1974b; Esposito et al., 1974). At the same time DNA synthesis can be completed in the absence of recombination. Mutations in the con 1, 2, 3 and 4 loci led strains to lose their ability to recombine

but did not affect premeiotic DNA synthesis in them (Fogel & Roth, 1974). Cell homozygous for the CSP1 mutation (which allow sporulation in α/α and a/a diploids) did not show any heteroalleic recombination when they returned to mitosis after they have completed DNA synthesis in sporulation medium (Hopper et al., 1975).

RNA and protein degradation

Sporulating cells of Saccharomyces cerevisiae show extensive breakdown of protein and RNA immediately after resuspension into sporulation medium. The maximum rate for both is reached roughly at the tetranucleate stage, before the depositions of the ascospore wall (Chen & Miller, 1968; Croes, 1967; Frank & Mills, 1978; Hopper et al., 1974; Klar & Halvorson, 1975). Addition of cycloheximide at any time during the process reduced the rate of degradation. The effects however differed for the two macromolecules. RNA degradation seemed to be affected drastically compared with protein breakdown (Klar & Halvorson, 1975; Magee & Hopper, 1974).

The need for new protein synthesis indicates the requirement for the synthesis of new enzymes or activators of enzymes. The activities of proteinases and RNases increase during sporulation (Betz & Weiser, 1976; Klar & Halvorson, 1975; Tsuboi, 1977). Three species of RNases have been detected using carboxymethyl-sephadex column chromotography from cell-free extracts of sporulating cultures. The activities of two remained constant during sporulation whereas one RNases III, showed increased levels of activity in depressed cells (Tsuboi, 1977). By the time sporulation is completed, 50 to 70% of the total

prexisting RNA has been broken down (Frank & Mills, 1978; Hopper et al., 1974) and a significant proportion of this total degradation may be attributed to the activity of RNase III. (Tsuboi, 1977).

Assays for intracellular proteinases indicated several-fold increases in the activities of proteinases A, B and C (Betz & Weiser, 1976; Klar & Halvorson, 1975). There is some degree of selective inactivation of individual proteins (Betz & Weiser, 1976; Klar et al., 1976). This may be due to the inherent susceptibility of the various proteins to degradation or compartmentalisation of proteinases and specific proteins (Klar & Halvorson, 1975; Sumrada & Cooper, 1978). Zubenko et al., (1979) have shown that most diploid Saccharomyces cerevisiae strains that are homozygous for prb 1 mutations in the structural gene for proteinase B are unable to sporulate normally; this implies that this enzyme is involved directly in the sporulation process.

In many instances, nutrient depletion seems to induce macromolecular breakdown to provide an intracellular pool for resynthesis needed for the cells to adapt the cells to the new environment. In sporulating yeast as well as Bacillus, the extensive breakdown appears to fulfill similar functions and to supplement the requirements for sporulation. This contribution is significant, the degradation occurs at the time of active periods of DNA replication, transcription and translation (Esposito et al., 1969). Sporulation of yeast is completely inhibited if these degradation activities are blocked (Klar & Halvorson, 1975; Magee & Hopper, 1974). An additional function of proteases may be to remove unwanted vegetative macromolecules and

probably reduce the amount of cytoplasmic constituents in preparation for the inactive state of the spores (Frank & Mills, 1978; Hopper et al., 1974).

Glycogen breakdown

The breakdown of glycogen begins at about the time it reaches its maximum rate of synthesis and when the rate of protein synthesis is maximal (Colonna & Magee, 1978; Hopper et al., 1974). Although the a/a and α/α cells accumulate glycogen, the concentration remains constant after reaching the peak of synthesis Colonna & Magee, 1978; Hopper et al., 1974; Kane & Roth, 1974). As for the breakdown of proteins and RNA, glycogen degradation depends on new protein synthesis (Magee & Hopper, 1974). Addition of cycloheximide at two to four hours after resuspension of cells into sporulation medium, inhibited its synthesis, whereas if the culture was treated at 6 hours, glycogen accumulated, apparently due to the failure of the cell to produce enzymes needed for breakdown of the reserve carbohydrate. So far, only one enzyme has been positively shown to be involved in glycogenolysis and this α-1-4 glucosidase is completely undetectable in non-sporulating or vegetative cells (Colonna & Magee, 1978).

The fate of the end products of glycogenolysis is not clear, since the rate of respiration is declining at the time of maximum breakdown (Hopper et al., 1974). Most likely some are incorporated into spore and ascus wall polysaccharides and a proportion supplements the depleting substrates to fuel energy production for the

completion of sporulation.

Development of resistance to diethyl ether

So far only one late event has been detected during sporogenesis : the maturing ascospores become resistance to diethyl ether. At a concentration of ether in which most of the vegetative cells are killed, 80-90% of the spores survive. (Dawes & Hardie, 1974). This acquisition may be due to the changes that occur in thickness and chemical composition of the spore walls. It has been shown that the spore coat is immunologically different from that of the ascus or cell walls (Snider & Miller, 1966) and is composed at least in part of protein.

CONTROL MECHANISMS INVOLVED IN THE REGULATION OF YEAST SPORULATION

It can be considered that the control mechanisms involved in the regulation of yeast sporulation operate at two levels, initiation and the subsequent control of the sporulation sequence. These two stages of controls depend on each other, precise coordination is necessary to ensure the successful completion of the process.

Control of initiation

Initiation of spore formation in yeast occurs at the stage in the life cycle in which the cells cease to undergo events leading to cell division and begin events that lead to meiosis and spore formation. Cell division on the one hand, and the meiosis and spore formation sequence on the other, are mutually exclusive processes to the extent that cells cannot bud and sporulate simultaneously. Once a cell has begun DNA synthesis in the cell division cycle it is effectively committed to continuing the cell division process (Hartwell, 1974). For this reason it is considered that at under appropriate conditions diploid cells 'decide' (or are 'switched') either to undergoing meiosis or to divide. This decision is controlled by three main factors:

1. Nutritional, both carbon and nitrogen sources.
2. The stage of development of the cell in the mitotic cycle.
3. The nature of the mating-type alleles presented in the cell.

Nutritional control

Sporulation in yeast is initiated when both carbon and

nitrogen sources are depleted. This requirement differs from that of Bacillus in which depletion of either sources is sufficient to initiate sporulation (Schaeffer et al., 1965). It reflects a more complex and stringent control of the initiation event in yeast.

Control of carbon substrates

The presence of hexose sugars such as glucose, fructose and mannose, at a concentration above 0.5% (w/v) in the sporulation medium inhibits sporulation completely (Fowell, 1967; Miller, 1957). Galactose however shows a less severe effect, due to the slightly different metabolic pathways utilised; though a fermentable carbon source, galactose does not repress the enzymes of the TCA cycle, the functioning of which is essential for sporulation (Kuenzi et al., 1974; Miller & Halpern, 1956; Polakis & Bartley, 1965).

The drastic effect of hexose sugars influences sporulation much earlier than the point of initiation. Comparisons of sporulation ability of cells harvested at different times during growth in glucose medium showed that cells from the exponential growth phase are unable to initiate sporogenesis on transfer to sporulation medium, whereas a population from early stationary phase is able to form a high percentage of asci (Fast, 1973). This is related to the ability of the population to switch to oxidative metabolism. Logarithmic phase cells are suppressed in their ability to do so, until at the end of the exponential phase, when glucose is depleted and the end product of fermentation, ethanol

begins to be utilised. This change preadapts the population to oxidative metabolism (Croes, 1967; Esposito et al., 1969).

The requirement for derepression of sporulation in yeast is markedly similar to the derepression of the synthesis of enzymes subject to carbon catabolite repressors. Apart from the TCA cycle enzymes, the importance of which has already been emphasised, there are several other enzymes which are under carbon catabolite repression. It is however, not known whether any of them is actually involved in sporogenesis. In order to determine the extent of the involvement of these enzymes, the ideal situation would be to isolate mutants in which sporulation is insensitive to repression by carbon catabolites. They can then be assessed for their ability to synthesise carbon catabolite repressed enzymes. In yeast, such mutants are not available at the present time. Kunst et al. (1974) have reported mutants of Bacillus subtilis defective in sacU^{h}, which were able to sporulate in the presence of glucose or an amino and mixture, at the same time these mutants produced excess extracellular sucrase and exoprotease. This phenotype was the result of a single gene mutation different from the CATA gene mutation isolated by Ito and Spizizen (1973) which showed a similar phenotype in respect of sporulation which has normal catabolite repression of enzyme synthesis. However Coote (1974) in attempts to correlate the control mechanism of inducible enzyme synthesis and repression of sporulation reached no firm conclusions, it remains possible that repression of sporulation and repression of inducible enzymes involve some common catabolite repressor effect, at the same time there is a fundamental

difference between the two processes.

The control of carbon catabolite repression on synthesis of enzymes has been studied extensively in E. coli (Gonzalez & Sheppard, 1976; Pastan & Adhya, 1976; Pastan & Perlman, 1970). It is based on the 'Operon model' of gene expression (Jacob & Monad, 1961), in which a repressor protein binds to the operator gene to prevent expression, when an inducer is present, it reacts with the repressor rendering it ineffective (Figure 4).

Removal of repressor, although necessary for operon expression is not sufficient for initiation of transcription. cAMP and cAMP receptor protein (CRP) are needed. The level of cAMP is controlled by the growth substrate. Glucose lowers the intracellular concentration of cyclic AMP by inhibiting its synthesis, effectively blocking initiation of mRNA synthesis (Peterkofsky & Gazdar, 1973).

Apart from acting as a positive regulatory element for turning on gene expression, cAMP can also act negatively as in the synthesis of a component of the adenylate cyclase complex (Potter et al., 1974) and several enzymes in the interconversion of glutamate and glutamine (Prusiner et al., 1972). Since cAMP and CRP complex is known to work at the gene level, it is likely that both the negative and positive effects of cAMP and CRP occur at transcription level.

The regulation of catabolite repression of enzyme synthesis in yeast has only been recently studied, and it has proved to be more complex than that in prokaryotes. Several mutations

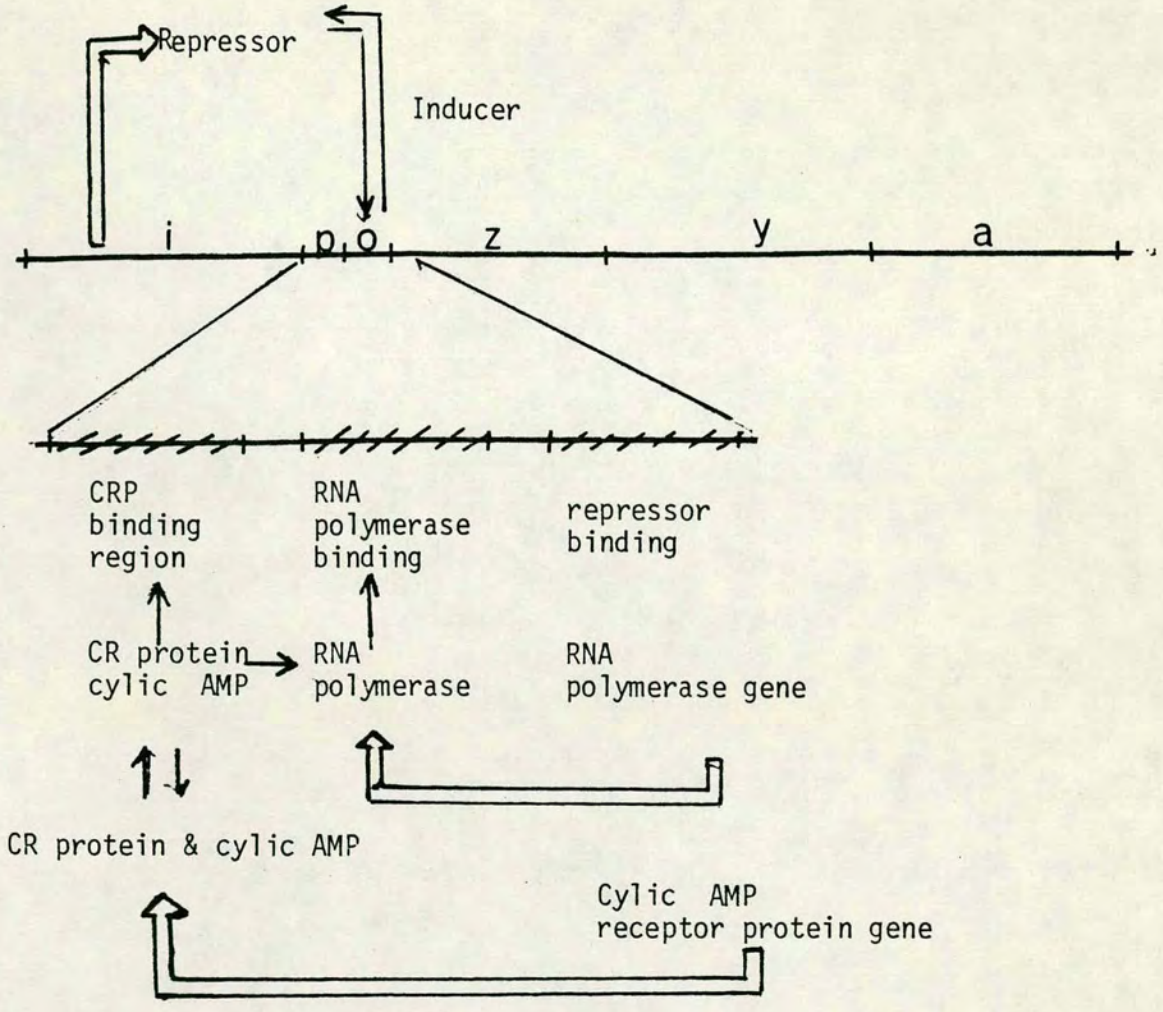


Figure 4: Scheme of cylic AMP action on the lac operon in E. coli. Heavy arrows indicate gene products and the light arrows indicate reactions that these proteins undergo. z, y and a are the three structural genes, i, p and o are the regulatory genes of the operon (Adapted from Pastan & Perlman, 1970).

have been isolated, such as MAL2, a mutation in the regulating gene for maltase synthesis (Zimmermann & Eaton, 1974) and cat 1-1, a single recessive mutation affecting several catabolite repressible functions (Zimmermann et al., 1977).

From studies of the cat 1-1 mutants and two other classes of mutants derived from it, Zimmermann & Scheel (1977) showed that at least two genes were involved in the regulation of carbon catabolite repression of enzymes synthesis in yeast, CAT1 and CAT2. They postulated that CAT1 forms a regulatory gene product which prevents the function of CAT2. The CAT2 gene product prevents depression of enzyme synthesis. Glucose blocks the expression of CAT1, resulting in a high activity of the CAT2 product, blocking enzyme synthesis. The CAT2 gene product has to be completely eliminated before initiation of transcription can occur. Another gene HEX1 has also been shown to be involved in carbon catabolite repression in yeast but this acts independently of CAT1 and CAT2 (Entian et al., 1977). The involvement of several unrelated genes indicated that carbon catabolite repression and derepression of enzyme synthesis is well regulated, not just on automatic response to the presence or removal of glucose.

There is also evidence of the involvement of cAMP in the regulating system. The intracellular concentration of cAMP was found to be low in S. carlsbergenesis under conditions of catabolite repression. The levels increased eight-fold during adaptation to maltose (van Wijk & Konijin, 1971). A specific and positive effect of cAMP has also been demonstrated in the induction of α-glucosidase in yeast protoplasts in the presence of 2% glucose and

4% maltose (Wiseman & Lim, 1974) cAMP may also be involved in the respiratory adaptation of S. cerevisiae. Severe inhibition by 10% (w/v) glucose can be overcome by adding cAMP (Fang & Butow, 1970).

Derepression of sporulation may be regulated by a similar system involving cAMP. In yeast the levels of cAMP fluctuate during sporulation (Hartig & Breitenbatch, 1977 ; Watson and Berry, 1976). The data from both groups indicate increases in the concentration of cAMP during active synthesis of macromolecules. Further evidence that suggested the repression of sporulation in yeast by glucose is related to the cAMP-controlled regulation system was shown by the ability of exogenously added cAMP to partially reverse the repression effect of glucose in sporulation medium. The action of cAMP was specific, no other related nucleotides gave any similar effect (Tsuboi et al., 1972). The results however were not confirmed by Hartig & Breitenbatch (1977). Moreover the main changes in cellular cAMP levels occur well after meiosis has been initiated.

Regulation by nitrogen sources

Cells introduced into sporulation medium are exposed to an imbalance situation in which energy is supplied but growth is limited due to the absence of a nitrogen source, and they go through an alternative pathway to cell division resulting in meiosis and spore-formation. Addition of certain nitrogen compounds has been shown to disturb this new path, causing marked depression of sporulation (Miller, 1963). Ammonium sulphate in excess of 2mM inhibited sporulation completely (Durieu-Trautmann & Delavier-Klutchko, 1977; Pinon, 1977). Even yeast extract seems to have a detrimental

effect. (Fowell, 1967; Haber & Halvorson, 1975). Some variation in these effects have been noted, these are probably due to the use of different strains and experimental conditions.

The understanding of the mechanisms by which nitrogen compounds affect sporulation is still at an embryonic stage. As mentioned earlier, triggering of sporulation in Bacillus species only needs the limitation of either carbon or nitrogen sources. In this respect the mediator of derepression of sporulation may differ to that in yeast. At the same time, as in carbon catabolite repression there is a remarkable similarity to the depression of enzyme synthesis. It is not surprising that most attempts in trying to elucidate the control mechanism of nitrogen repression have been directed towards the understanding of enzyme synthesis.

There are several enzymes in yeast whose synthesis is subjected to nitrogen repression. These include arginase, allantoinase, $NADP^+$-dependent glutamate dehydrogenase and urea amidolyase (Dubois et al., 1973). Generally these are also inducible enzymes (Arst & Cove, 1973; Wiame, 1971). Most authors agree that ammonium ion is the main effector of repression. At the same time glutamine and asparagine can be as effective in repression as $NH4^+$ (Dubois et al., 1977: Haynes, 1974). Rapid interconvertibility of glutamine and ammonium ions has made identification of the primary mediator signal difficult.

Bossinger et al., (1974) however reported only a mild repressive effect of $NH4^+$ on allantoinase and arginase activities compared

with trasaminatable amino acids such as serine, glutamine and asparigine. They concluded that the observed repressive effect of $NH4^+$ is due to its metabolism to transaminatable amino acids.

Using mutants lacking in $NADP^+$-dependent glutamate dehydrogenase, Dubois et al., (1977) demonstrated the primary role of $NH4^+$. The mutation caused poor growth on medium with ($NH4^+$) as nitrogen source (Grenson & Hou, 1972). It also allowed the derepression of several $NH4^+$ - repressible enzymes such as arginase, urea amidolyase, allantoinase and several permease functions. Addition of glutamate restored growth because the block in nitrogen assimilation was bypassed but the repressed level of arginase was not restored in an operator - constitutive mutant (Dubois et al., 1974). Based on this evidence, a scheme was proposed where by $NH4^+$ is the signal received by the $NADP^+$ - dependent glutamate dehydrogenase, the resulting complex acts in concert with another factor to mediate repression possibly at the gene level. The authors (Dubois et al., 1977) also postulated another mode of nitrogen control involving glutamine as a signal. The product of the gene Gnr R formed a complex with glutamine with the possible participation of glutamine synthetase to effect repression of certain enzymes. A regulatory role of glutamine synthetase in the regulation of nitrogen repression of enzyme synthesis has been proposed in Klebsiella aerogenese (Prival et al., 1973).

In B. megaterium, a similar role of glutamine synthetase in the regulation of sporulation has been postulated (Reysset & Aubert, 1975). Mutants defective in this enzyme showed poor sporulation

and the two phenotypes always coreverted. These authors also isolated a rare mutant which was only affected in the enzymic activity of glutamine synthetase but in which sporulation was normal. It was proposed that glutamate synthetase may be the receptor of a low molecular weight effector from the purine biosynthetic pathway and the complex acted in the regulation of sporulation. So far no involvment of glutamine synthetase has been observed in yeast.

Despite some controversy over the role of ammonium ion in the repression of enzyme synthesis, Pinon (1977) indicated that $NH4^+$ was responsible for the control of repression of sporulation. The effect was mimicked by methylamine, a non metabolized analogue of ammonia. Glutamine showed a similar effect since it acted as a source of ammonia. The effect was directly acting on the sporulation mechanism. $NH4^+$ at a concentration which completely inhibited sporulations did not affect respiration or the level of ATP in the cell. The existence of separate control mechanisms between nitrogen repression of enzyme synthesis and sporulation has also been illustrated in the study of derepressed sporulation (spd) mutants (Dawes, 1975). These mutations allowed sporulation to occur in the presence of high concentrations of nitrogen sources which usually suppressed sporulation but it did not affect the regulations of nitrogen repressible enzyme synthesis (Kinnaird & Dawes, 1979).

Cell cycle control

The ability of cells to initiate sporulation seems to be confined only to the G1 phase of the cell division cycle (Hirschberg & Simchen, 1977; Shilo et al., 1978). Cells in different phases of the cycle have to complete mitosis and reach this crucial point before they attain competence to initiate sporulation. (Haber & Halvorson, 1972; Hischberg & Simchen, 1977). Daughter cells which have not undergone mitosis do not seem to be able to initiate sporulation (Sando et al., 1973). The control of this periodic capacity is not known at the present time. Haber & Halvorson (1972) concluded that at other stages of the cycle sporulation may be limited by the unavailability of one or more essential component which either fluctuated periodically throughout the cell cycle or only reached the threshold level required for initiation of sporulation in cells of greater volume. Most likely it indicated the presence of control mechanisms for the expression of sporulation-specific functions which would only be inducible during a limited period of the mitotic cycle.

The decision by the cell to undergo either mitosis or meiosis is influenced by the nutritional environment. The mitotic cycle is continued if growth factors are abundant. Evidence from several laboratories indicated that initiation of the mitotic cycle takes place very close to, if not at the same point as, the initiation of meiosis (Hartwell, 1974; Shilo et al., 1978; Vezinhet et al., 1979). The two cycles also share several other common features. Both involve new DNA synthesis (Croes, 1967), the duplication and segregation of spindle plaques, and nuclear division (Moens and Rapport,

1971a; Hartwell, 1974). The nuclear events that formed many of the "landmarks" in both meiosis, mitosis were also fundamentally similar, and the same gene products have been found to mediate these processes. Thirteen out of the twenty cdc genes that have been tested were found to be essential also for both meiosis and sporulation (Simchen, 1974). As expected these essential genes are defined by cdc mutations that affected many of the common features, such as plaque duplication and separation, DNA synthesis and nuclear division The other seven mutations that did not effect sporulation in homozygous diploid strains were all concerned with the diagnostic cell division landmarks that are not involved in meiosis or spore formation, such as bud emergence and cytokinesis.

Although mutants homozygous for each of the thirteen cdc mutations have been shown to be affected in meiosis, their stages of arrest in the meiotic cycle have not been investigated fully, except for the case of cdc4. In the mitotic cycle, cells carrying cdc4 were arrested in the double plaque configuration however they do not initiate DNA synthesis (Hartwell, 1974). In meiosis, the mutation showed at least three phenotypes at the restrictive temperature depending on the time of transfer from the permissive to the restrictive conditions (Simchen & Hischberg, 1977). The author suggested that the three recognisable terminal phenotypes indicated that the function of this gene was required in at least three different points in the process, DNA initiation, plaque separation and spore formation after the second meiotic division. This led to the conclusion that the DNA replication defect (whether in meiosis or in vegetative cells)

was only a secondary result of the cdc4 defect. The normal CDC4 allele may be involved in chromosome condensation, which may in turn be required for DNA synthesis initiation, for plaques separation prior to the first and second meiotic division and for the packaging of nuclei into spores after the second meiotic division.

Mating type control

Haploid cells of Saccharomyces cerevisiae are either of a or α mating type. These two cells conjugate on contact to give diploids which are heterozygous at the mating type locus, and this is a necessary prerequisite for meiosis and spore formation. (Friis & Roman, 1968; Roth & Lusnak, 1970). Any combination of the mating type alleles with at least one a and one α allele allows sporulation to occur, regardless of the ploidy state of the cell (i.e. a/a/α cells sporulate; a/a/a cannot).

The fact that haploids, and cells homozygous at the mating-type locus are unable to sporulate, suggests that products from both alleles are required to derepress sporulation, most probably by acting at the level of transcription of certain genes, as indicated by the abnormal a* allele isolated by Kassir & Simchen (1976). When a* cells were crossed to normal α cells, the resulting diploids (a*/α) behaved as α maters. They also failed to sporulate due to the presence of α products or lack of a normal a/α complex. The a and α functions once synthesised, are not limited to the nucleus but can act through the cytoplasm. This was demonstrated by the mating of kar1 haploids (Karyogamy defective mutants) to homozygous

diploids of the opposite mating type. The resulting zygotes are heterokaryons which are able to sporulate, producing six spores. Genetic analysis showed that the diploid nucleus underwent normal meiosis resulting in four haploid spores of the same mating type. The other two spores carried genotypes attributed only to the kar 1 haploid (Klar, 1979).

Although the a/α gene products seem to be the central control of repression of initiation of sporulation, other genes are also involved which under certain conditions can by pass the a/α control. For example in CSPI mutants sporulation occurs in 25% of the population of α/α diploids. (Hopper et al., 1974). The authors put forward two distinct hypotheses about the a/α dependent gene regulation that could explain the nature of the CSPI diploids. Firstly, the CSPI mutation probably partially corrected the pleiotropic defect of the α/α strains, resulting in a low level of expression of genes dependent on the a/α control. The second hypothesis predicted that the defectiveness of CSPI was due to a single step block in meiosis. The gene functions responsible for this step were expressed at low, leak-through level in α/α CSPI diploids, as a result, a few cells circumvent the block and go on to sporulate. The CSPI probably exerted its effect through an intermediate gene product in the normal pathway between the a/α mating type gene and the sporulation genes (Hopper & Hall, 1975).

Two other mutations have been reported to allow a/a or α/α cells to sporulate, the sca gene (Gerlach, 1974) and the rme gene (Kassir & Simchen, 1976). Unlike the CSPI which was a dominant mutation, these two were recessive. They differed however in their map

location position, the *sca* gene was unlinked to the mating-type locus, while the *rme* gene was located on chromosome III to the left of the mating-type locus and linked closely to the centromere.

Control of subsequent development

Successful initiation does not ensure that the subsequent development of meiosis and spore formation can occur without interrruption. The process is further monitored by a complex regulatory system which is partly related to the control of initiation as shown by the prolonged sensitivity to carbon and nitrogen levels in the medium and mutants that by pass the mating-type control discussed earlier.

Control by specific genes

In the sequence of meiosis and sporulation events that has been established, initiation is followed by a distinct premeiotic DNA synthesis, accompanied by a high level of genetic recombination (Hopper et al., 1974; Roth & Lusnak, 1970). DNA synthesis has a much longer duration compared to mitotic DNA synthesis (Williamson et al., 1980). Autoradiagraphic data indicated that the slower rate is due to the reduction in number of replication initiation points while the rate of chain growth remained the same (Callan, 1972). Roth (1973) suggested that these differences in overall rate and patterns of DNA synthesis indicated that unique or modified gene products distinct from those utilised during vegetative growth are required during meiosis and subsequent spore development. He characterised recessive mutants designated mei 1, 2 and 3 which affected different gene products involved in meiotic DNA synthesis and recombination. The vegetative growth phase of these mutants was not affected.

Meiotic DNA synthesis also seems to depend at least on

parts of the regulatory control of the mitotic cycle. Studies on temperature-sensitive mutants (cdc mutants) showed that a number of the genes that are involved in mitotic DNA synthesis are also operative in meiotic DNA synthesis (Hartwell, 1974; Simchen, 1973). These included those identified by the cdc 4, cdc 7 and cdc 28 mutations which all affect initiation of DNA synthesis.

Isolation and characterisation of mutants defective in functions specific to meiosis and ascospore development further indicated the involvement of specific genes in the control of meiosis and spore formation. Both recessive (spo) and dominant (SPO) temperature-sensitive mutants have been isolated (Esposito & Esposito 1969; Esposito et al., 1972) and complementation studies indicated that approximately 50 loci (a fairly rough estimate) code for functions indispensible for sporulation. (Esposito et al., 1972). Among the loci that have been isolated lesions in spo 7, 8, 9 , 11 and a dominant mutation SPO98 prevented meiotic DNA synthesis at the restrictive temperature (Esposito & Esposito, 1973; 1974a). Electron microscopy showed that at the restrictive temperature they terminated before the duplication of the spindle pole body, (Moens, 1977). Cells homozygous for spo 11 however exhibited abnormal meiosis despite the absence of DNA synthesis (Moens et al., 1977).

Spindle pole body duplication follows premeiotic DNA synthesis (Byers & Goetsch, 1975; Esposito & Esposito, 1978). Premeiotic DNA synthesis, commitment to recombination and synaptonemal complex formation are not absolute requirements for spindle pole body duplication or aspects of meiotic prophase as shown by spo 11, which

undergoes an abnormal first meiotic division without any apparent DNA synthesis (Moens et al., 1977). Diploids homozygous for either spo 1 or spo 10 completed events before the spindle pole body duplicated, but were unable to proceed further at the restrictive temperature (Moens et al., 1974). Temperature-shift experiments with spo 1 indicated that the SPO 1 gene function was expressed before spindle pole body duplication and the products seemed to be required even after the stage defined by the phenotype terminated.

The execution of the first meiotic division requires the product of the SPO 2 gene. Diploids homozygous for the spo 2 mutations were not only defective in the first meiotic division, but the abnormality recurred at the second meiotic division, resulting in four anucleate closed prospores and four free nuclei (Moens et al., 1974) as was the case for SPO 1, temperature-shift experiments indicated that the SPO 2 gene products were needed well before the onset of the first meiotic division (Esposito et al., 1970). Most probably SPO 2 encoded a structural component of the nuclear membrane and it affected the properties of the nuclear membrane or cellular constituents interacting with it (Meons et al., 1974).

Three other mutations, spo 3, spo 4 and spo 5 affected the beginning of the second meiotic divisions (Moens et al., 1974; Esposito & Esposito, 1978). The SPO 3 gene is expressed and its function completed at or just prior to the second divisions; it is probably required for the incorporation of haploid nuclei into spores (Esposito & Esposito, 1978). Diploids homozygous for defects in any of the above three loci, had lost co-ordination of the prospore wall

growth and closure and nuclear budding. The prospore walls frequently became prematurely detached from the spindle pole body and the nuclei withdrew from the prospore wall cavity. The typical terminal phenotype consisted of an ascogenous cell containing a large nucleus arrested in early nuclear budding and immature prospores containing portions of the nuclei (Esposito & Esposito, 1978).

From the phenotype of homozygous diploids and the behaviour of the other spo mutants which demonstrated several features of sporulation, Esposito & Esposito (1978) concluded that: Meiosis I and meiosis II are under common genetic control, prospore wall formation, growth and closure are independent of the entry of the nucleus into the prospore; and, ascospore wall maturation i.e. the deposition of materials between the membranes and the prospore wall is, in part an autonomous function of the nucleated prospores.

Nutritional control

The inhibitory effects on sporulation of high levels of glucose and nitrogen only cease when premeiotic DNA synthesis and replication are completed (Esposito & Esposito, 1974a; Simchen & Hirchberg 1977; Simchen et al., 1972). The sensitive period prior to this completion was suggested to be the period of active transcription of "new" sporulation specific genes. If this is so, once the essential gene products were available, they seemed to be stable enough for the subsequent development to proceed. It could, however, also be the period in which modification of certain parts of the transcription and/or translation machineries of the cells takes place, so that the nutritional environment no longer has any effect on gene expression. This, to some extent begs the question of whether or not there is any unique gene expression at all.

Co-operative control of the mating type locus and a complete diploid genome

The mating-type locus exerts a stringent control over the initiation event of sporulation. Its presence however is only sufficient to initiate DNA synthesis and probably a certain degree of recombination. Completion of other events needs the full complement of the diploid nucleus. Haploids carrying disomic chromosome III with a/α constitution at the mating-type locus were able to initiate pre meiotic DNA synthesis when exposed to sporulation conditions. They were not, however, able to show any further development (Roth & Fogel, 1971). At the same time, diploids homozygous for either allele at the mating-type locus are blocked in the initiation of sporulation. From these results it was suggested that the a/α gene product or complex is involved in the functioning of the necessary genes in the diploid nucleus for subsequent progress of sporogenesis.

The inadequacy of the a/α function was further illustrated by a mutation which provides the a and α products by allowing the expression of the silent mating-type information (Hicks et al., 1977). Strains carrying this mutation failed to respond to the a and α factor as typified by a/α diploids and only exhibited incipient sporulation similar to that in the haploids disomic for chromosome III (Klar et al., 1979). Heterokaryons constructed by mating the kar 1 (karyogamy-defective) haploids and standard haploid strains were also unable to sporulate. In heterokaryons with diploid nuclei, the latter must be producing additional factors during normal meiosis which influenced the haploid nucleus to undergo spore formation (klar, 1980).

STUDIES OF PROTEIN CHANGES DURING SPORULATION OF SACCHAROMYCES CEREVISIAE

Genetic data have indicated that about 50 loci code for functions indispensable to sporulation in Saccharomyces cerevisiae (Esposito et al., 1972). Despite these findings, few biochemical changes have been shown to be specifically involved in sporulation (Croes, 1967; Esposito et al., 1969; Haber et al., 1974; Tingle et al., 1973). Little is known about the role of gene expression in the process, its timing, or its regulation in molecular terms, and further progress depends on the identification of more sporulation-specific events. Several attempts to distinguish specific protein changes by pulse-labelling sporulating cultures with amino acids and subsequently examining their complement of polypeptides by one-dimensional (Hopper et al., 1974) or two-dimensional (Petersen et al., 1979; Trew et al., 1979) polyacrylamide gel electrophoresis have met with little success. Both non-sporulating and sporulating cultures showed similar changes under sporulation condition, thus these changes probably resulted from the shift to the starvation condition, and were not specifically associated with sporulation.

Under usual conditions used to induce sporulation pH of the medium increases rapidly at the early stages of sporulation (Croes, 1967) and this has been recognised as a factor that lowers cellular permeability for amino acids (Mills, 1972). Therefore, some of the results observed in pulse-labelling studies may have been due to differential uptake of added amino acids by different sub-populations of cells in sporulating cultures; the bulk of labelled amino acids may be taken up by vegetative cells, or those at early stages in

sporulation.

In view of some of these factors that may directly or indirectly mask the true pattern of protein synthesis alterations during sporulation a preliminary study of the uptake of labelled precursors by sporulating cultures was undertaken. The findings from these experiments highlighted the problems of labelling such cultures. Hence, a modified approach to labelling was employed to identify sporulation specific changes in polypeptides during sporulation. Instead of labelling at intervals, the cellular proteins were labelled continuously throughout presporulation growth in the presence of $^{35}SO_4^{2-}$, and then two-dimensional gel electrophoresis was used to analyse the labelled polypeptides of cells transferred to sulphur-free sporulation medium. This technique has the advantage that it enables the detection of changes arising from modifications to existing labelled proteins as well as from de novo synthesis. It does not, however, allow direct distinction between these two possibilities.

Using this approach Wright & Dawes (1979) were able to detect specific changes during sporulation. 11% of the prominent polypeptides detected showed changes (Figure 5).

This thesis reports the extension of these experiments to examine whether the changes occurred in any specific pattern and time during sporulation, in order to identify if possible, the extent to which protein changes were regulated and to provide a basis for more detailed study of the regulation of gene expression (or protein modification).

Sporulation mutants were isolated and characterised both biochemically and genetically to study the relationship between protein changes occurring during sporulation and the morphological events and to determine whether these protein alterations were part of a dependent sequence of events. Preliminary studies were begun using cycloheximide as an inhibitor of protein synthesis (on cytoplasmic ribosomes) to determine whether any of the sporulation-specific protein changes were indepedent of nuclear gene expression. This is a first step towards assigning changes to modification events rather than to gene expression.

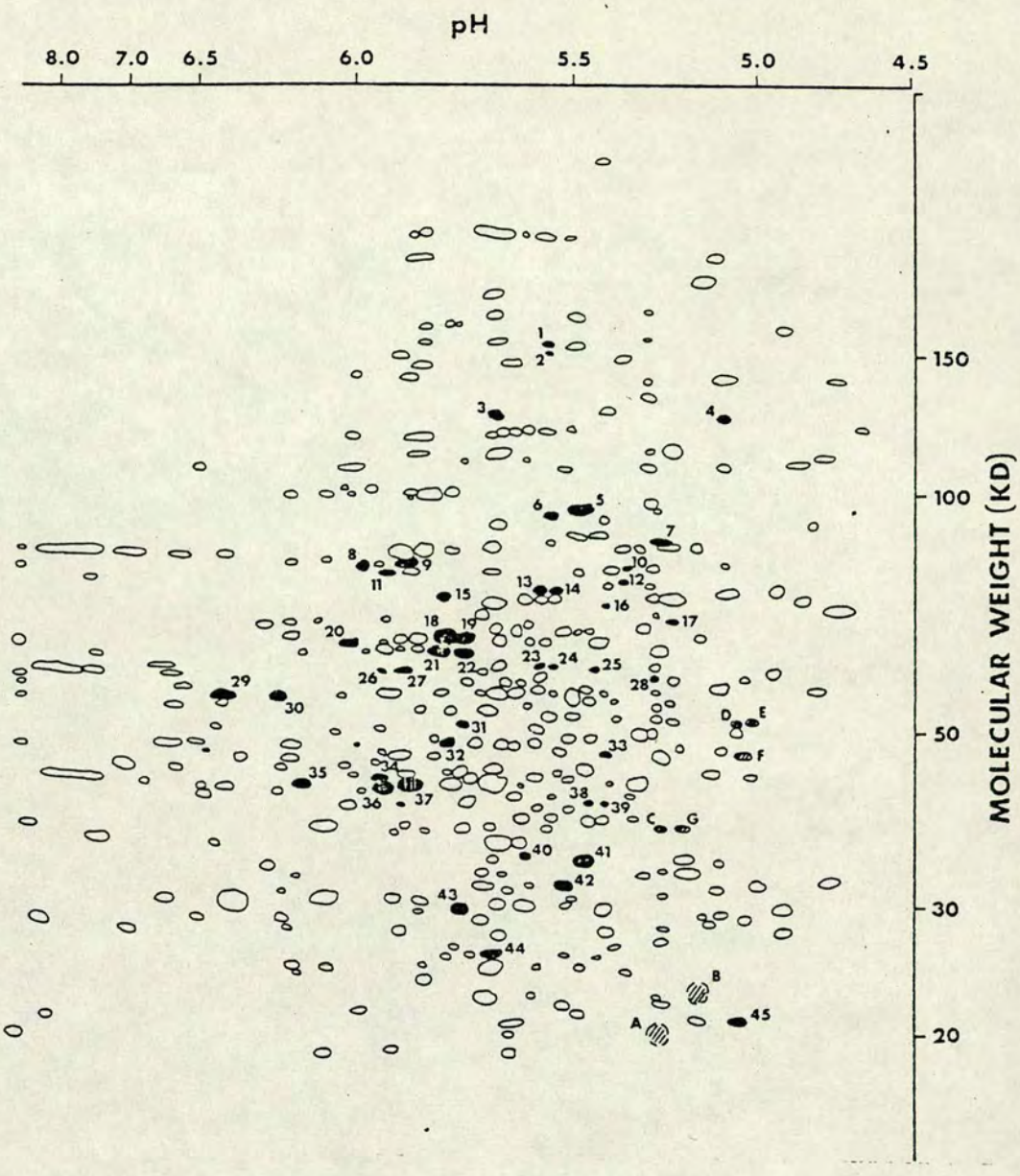


Figure 5 : Composite diagram of the most prominent polypeptides detected by autofluorography of[^{35}S]- labelled yeast proteins (Wright & Dawes, 1979). Numbered spots correspond to polypeptides that showed changes during the 24h experiment. A-E indicate polypeptides detected by ^{32}P-orthophosphate labelling only.

MATERIALS AND METHODS

Organisms

Strains of Saccharomyces cerevisiae and E. coli used in this study are presented in Table 1. Definitions of genetical abbreviations are listed in Table 2.

Culture conditions

Complete medium containing 2% (w/v) bactopeptone, 1% (w/v) yeast extract and 2% (w/v) glucose (YEPD) was used for routine growth and maintenance of stocks at $0^{o}C$. (Solid medium was supplemented with 2% (w/v) agar). All strains were cloned before use. Where required the glucose was substituted by 2% (w/v) potassium acetate (YEPA), 3% (v/v) glycerol (YEPG) or 2% (w/v) glactose (YEPGAL).

Defined minimal medium contained: Difco yeast nitrogen base without $(NH_4)_2SO_4$ and without amino acids (1.5 gl^{-1}) to provide trace elements, salts and vitamins; nitrogen source usually ammonium sulphate (5.0 gl^{-1}); any carbon source (2% w/v) and auxotrophic requirements (20$\mu g.ml^{-1}$) added where necessary.

Labelling of cell proteins with $^{35}SO_4^{2-}$ was done in low sulphate medium (based on Mitchison, 1970). ($^{35}SO_4^{2-}$ and all other labelled precursors used were obtained from the Radiochemical Centre, Amersham). The components of low sulphate medium and their concentration are listed below.

Component	Final concentration/litre
Galactose (as a derepressing carbon source)	20g
Sodium Acetate	1g
Potassium Chloride	1g
Magnesium Chloride	500mg
Calcium Chloride	10mg
Sodium Chloride	10mg
Ammonium Chloride	5g
Potassium hydrogen Phthalate	3g
Sodium hydrogen Phosphate	1.8g
Inositol	2mg
Nicotinic Acid	2mg
Calcium Pantothenate	400µg
Biotin	400µg
Riboflavin	200µg
Pyridoxine	400µg
Boric acid	500µg
Manganese Chloride	461µg
Zinc acetate	305µg
Sodium Molybdate	215µg
Potassium Iodine	100µg
Copper Chloride	27µg
Citric acid	1µg
Ferric Chloride	200µg
Folic acid	2µg
p-Amino Benzoate	200µg
Thiamine Hydrochloride	400µg
Amino acid (added as required)	20mg

Table 1

Saccharomyces cerevisiae

STRAIN	ORIGIN	GENOTYPES	REMARKS
JW1	Crosses of spores of spd strains derived from S41	a ARG4-17 his5-2 LEU2-1 lys1-1 spd1-1 TRP1-1 URA3-1 α arg4-17 his5-2 leu2-1 lys1-1 spd1-1 trp1-1 ura3-1	spd 1 strain good sporulation
JW2	Isolated by UV mutagenesis of JW1	a ARG4-17 his5-2 LEU2-1 lys1-1 spd1-1 TRP1-1 URA3-1 a arg4-17 his5-2 leu2-1 lys1-1 spd1-1 trp1-1 ura3-1	Non-sporulating isogenic to JW1 used as control
Y174	Brandeis University	α adel arg6 his1 thr3 trp2 ura3	his1 marker showed good NTG+ induced reversion
Y323	Brandeis University	α adel his2 leu1 met14 trp1	trp1 marker showed good NTG induced reversion
S41	H.O. Halvorson Brandeis University	a HO arg4-1 cyh1 α HO arg4-1 cyh1	A homothallic diploid parent of 135.11B
14Ax17C	Built from Y174 and Y323	a adel1 his1 trp1 α ADE1 his1 trp1	good sporulation used in NTG experiments

+ N-methyl-N'-nitro-N-nitrosoguanidine

STRAIN	ORIGIN	GENOTYPES	REMARKS
D609-28C	Cold Spring Habor collection	a arg4-17 his5-2 lys1-1 trp1-1 aro7-1	Ochre and amber suppressible markers
135.11B	S41	a HO arg4 met14 ura3 α HO arg4 met14 ura 3	Good sporulating diploid
XN129	Isolated by UV mutagenesis of 135.11B	a HO arg4 met14 spo52 ura3 α HO arg4 met14 spo52 ura3	Asporogenous strain
XN02	Built from IWD haploid collection good sporulation UV converted at mating type locus	α ARG4 LEU3 MET14 trp1 α arg4 leu3 met14 trp1	Crossed to XN129
XN011	Derived from XN129	a HO arg4 spo52 α HO arg4 spo52	Asporogenous, grew in low sulphate medium, XN129 did not.

STRAIN	ORIGIN	GENOTYPES	REMARKS
69.10C	Isolated by EMS mutagenesis of *spd1* diploid	$\frac{a\ HO\ arg4\ spo50\ ura3}{\alpha\ HO\ arg4\ spo50\ ura3}$	Asporogenous strain
5E-D	Isolated by UV mutagenesis of JW1	$\frac{\alpha\ ARG4\ his5\text{-}2\ LEU2\text{-}1\ lys1\text{-}1\ spd1\text{-}1\ TRP1\text{-}1\ URA3\text{-}1}{\alpha\ arg4\ his5\text{-}2\ leu2\text{-}1\ lys1\text{-}1\ spd1\text{-}1\ trp1\text{-}1\ ura3\text{-}1}$	Crossed to 69.10C
Y204	The Berkeley Yeast Genetic Stock Culture Collection	*a ade5*	Tester strain
Y209	"	*α ade5*	Tester strain
rad bearing strains	Donner laboratory University of California Berkeley	*a* and *α radl* to *rad57*	UV radiation sensitive strain
AH22	From Hinnen *et al.*, Cornell University	*a leu2-3 leu2-112 his4 can1*	Recipient strain for yeast transformation

STRAIN	ORIGIN	GENOTYPES	REMARKS
60 x 61	IWD collection	a HO ade2-40 ARG4-1 CAN1 CYH2 his4-166 α HO ade2-119 arg4-1 can1 cyh2 his4-239	ade and his heteroallelic markers used to estimate meiotic recombination and segregation

E. coli

STRAIN	ORIGIN	GENOTYPES	REMARKS
HB 101	Dr. M. Smith - Molecular Biology, Edinburgh	hsdR$^-$ hsdM$^-$ recA13 supE44 (su^{2f}) lac Z4 leuB6 proA2 thri-1 (B1$^-$) SM^R	Strain used to propagate plasmids, sensitive to ampicillin

Table 2

Key to Abbreviations

Saccharomyces cerevisiae

a, α	alleles of the mating-type locus
ade	adenine auxotroph
arg	arginine auxotroph
can	canavanine resistance
cyh	cycloheximide resistance
his	histidine auxotroph
HO	gene conferring homothallism which cause specific conversion of mother cells to the opposite mating-type; self-mating results in homozygous diploids.
leu	leucine auxotroph
Spd	mutation conferring derepressed sporulation
Spo	mutation conferring asporogeny
trp	tryptophan auxotroph
ura	uracil auxotroph

E. coli

	Phenotypic trait affected
hsdM$^-$	host modification activity, DNA methylase
hsdR$^-$	host restriction activity, endonuclease R
lac Z	β-D-glactosidase (Ec 3.2.1.23)
leu B	2-isopropylmalate dehydrogenase (EC.1.1.1.85)
pro A	block before L-glutamate semi aldehyde
rec A	general recombination; repair of radiation damage; induction of phage lambda.
sup E	Suppressor of amber (AUG) mutation
thi-B	Thiaminephosphate pyrophosphorylase (EC.2.5.1.3)

Sporulation conditions

Solid medium for sporulation contained per litre : potassium acetate 20g, glucose.5g, yeast extract 2.2g and agar 20g. Sporulation in liquid medium was done according to the method of Fast (1973). Cultures were grown to a turbidity of 1 (600nm) on YEPA medium centrifuged rapidly, then resuspended in 2% (w/v) potassium acetate (pH 7.0).

All media were sterilised by autoclaving for 20 min at 103.4kPa (120^{o}C).

Cells were routinely grown and sporulated at 30^{o}C, liquid cultures were aerated by mechanical shaking.

Culture conditions for E. coli

E. coli was routinely grown in L-broth containing per litre: Difco Bacto Tryptone, 10g; Difco bacto yeast extract, 5g; Sodium chloride, 5g; glucose, 1g, adjusted to pH 7.2) or L-agar (Difco Bacto Tryptone, 10g; Difco Bacto Yeast extract, 5g; Sodium chloride 10g; Difco agar, 15g per litre adjusted to pH 7.2). Incubated at 37^{o}C. Liquid cultures were aerated by mechanical shaking.

Growth measurement

Growth of cultures was determined by measuring turbidity at 600nm. Since the curve of cell concentration versus turbidity is non-linear above, 1.0, samples were diluted with water where necessary to a turbidity in the range 0.5 to 1.0.

Estimation of sporulation frequency

Percentage sporulation was estimated by direct counting of asci and vegetative cells in the phase contrast microscope (x400) in several non-overlapping fields. No distinction was drawn between buds and mother cells.

Uptake and incorporation of labelled amino acids for density-gradient centrifugation

Separate uptake and incorporation studies were done using either L-[^{14}C] phenylalanine or L-[^{14}C] arginine. The sporulating culture (3ml) was either labelled directly by adding the labelled amino acid as described below, or it was first centrifuged at 300g for 2 min and the cell pellet was resuspended in the same volume of either 2% (w/v) potassium acetate adjusted to pH6. or 2% (w/v) potassium acetate buffered to pH6 with 0.2M-morpholinopropanesulphonic acid (MOPS). The resuspension medium was warmed to 30^{o}C before use and contained L-[^{14}C] arginine at 5μCi ml^{-1} and 5μg ml^{-1} or L-[^{14}C]-phenylalanine at 5μCi ml^{-1} and 5μg ml^{-1}. After a pulse of 10 min, a sample (1ml) was gently layered over the Urografin (Schering Chemical Ltd., Burgess Hill, West Sussex) or Percoll (Pharmacia Fine Chemicals, Sweden) gradient and centrifuged immediately. Under these conditions uptake refers to the amount of ^{14}C retained by the cells after centrifugation through the gradient. Samples (10μl) from each fraction obtained from the gradient were transferred directly into scintillant (1ml Instra-gel, Packard Instrument Co.) and counted. Incorporation into trichloroacetic acid (TCA) precipitable material was measured by taking 0.1ml samples from each fraction in 10% (w/v) TCA at 0^{o}C and after 30 min, filtering on nitrocellulose filters (0.45 μm pore size).

The filters were counted in 3ml of 0.4% (w/v) 2,5 - diphenyl - oxazole in toluene.

Density-gradient centrifugation

Linear Urografin density gradients were prepared in 16.5ml capacity polycarbonate centrifuge tubes (MSE Code 0178) over the density range specified, usually within the limits of 1.13 to 1.22 g cm^{-3}. Best separations were achieved using a density range of about 0.06gcm^{-3}, and since these were quite shallow gradients, experiments were often carried out using several gradients with different ranges and that which yielded the best separation was used for analysis. Samples (1ml) of sporulating cultures (containing about 10^8 cells) were gently layered on the gradients and the tubes were centrifuged at 4500 g (max) for 10 min in an MSE Super-Minor centrifuge with swing out rotor. Fractions (0.3 ml) were obtained by piercing the tube and collecting from the bottom of the gradient. Percoll gradients were prepared in similar tubes over the density range of 1.0 to 1.13 g cm^{-3}. The same amount of sample was used and tubes were centrifuged as for Urografin gradients.

Density estimation

The density of each fraction was determined by measuring its refractive index and comparing this with a standard curve prepared from Urografin solutions or Percoll solutions of known density.

Cells concentrations and viability

The concentrations were estimated in terms of the turbidity of samples at 600nm. Samples were diluted 10 or 20-fold to reduce the effect of refractive index changes due to the presence of Urografin or Percoll. Cell viability was estimated by plating appropriate dilutions of the fractions on YEPD plates. After incubation of these plates for at least 3 days at 30°C it was possible to score for the red/pink character displayed by the ade 2 auxotrophs, as well as to estimate the total viable count.

Commit ment to recombination and segregation

Fractions were diluted and appropriately plated on defined complete medium plates lacking histidine supplement and the plates were incubated at 30°C for at least 5 days. Colonies were counted and the ratio of histidine-independent to total viable units was taken as an indication of the extent of commitment to intragenic recombination (Esposito & Esposito, 1974). Meiotic segregation was estimated by the proportion of red/pink sectored colonies from the YEPD plates used to estimate the total viable count. The sectored colonies were presumed to have arisen either by recombination or segregation within an ascus, and were scored as a single viable unit. In the case of diploids with a heterozygous dominant marker, fractions were diluted and plated on defined complete medium plates lacking the appropriate requirements. Meiotic recombination were scored by the proportion of sectored and colonies which were dependent on the requirement.

ONE DIMENSIONAL ELECTROPHORESIS OF PROTEIN EXTRACTS

Preparation of protein extracts

Protein extracts were prepared by the method of Laemmli (1970). 2ml of samples (0.5×10^8 cells ml^{-1}) was harvested and washed once in Tris-HCl, pH 6.8, then resuspended in 0.4 ml in homogenising buffer (0.0625 M Tris-HCl, pH6.8, 5% β-mercaptoethanol, 3% (w/v) sodium dodecyl sulphate (SDS); 0.03 mg ml^{-1} phenylmethylsulfonyl-fluoride (PMSF) was added immediately before use) and 0.5ml glass beads (0.45 μm in diameter) was added. The mixture was homogenised in a Brown homogeniser for 20s. The glass beads were removed by centrifugation through a separator, and the cell lysate collected was immediately boiled for 3 min, and stored at -20°C.

Electrophoresis of protein extracts

All glassware was thoroughly cleaned by standing in chromic acid, washed in tap water and distilled water and oven-dried. Cassettes were prepared by clipping together two glass plates separated by perspex spacers with a little vaseline on either side of the spacers.

The following gel solutions were made:

	15%	7%
Resolving gel buffer (1.5M Tris HCl, pH6.8, 0.4% SDS)	4.0ml	4.0ml
Acrylamide solution (29.8 acrylamide, 0.2g Bis-acrylamide to 100ml of water)	8.0ml	3.7ml
Water	4.0ml	8.3ml
	16	16

Both solutions were thoroughly mixed and degassed.

The solutions were loaded in a gradient maker with the 15% acrylamide solution in the mixing chamber. To each chamber, 20µl TEMED and 20µl Ammonium persulphate (10% solution) were added; 30µl of persulphate was added to the 15% solution. After mixing they were run into the cassette over 15-20 minutes to within 25mM of the lip of the front plate. The surface of the gel was overlayed with water-saturated butanol. Polymerisation was allowed to proceed overnight.

Immediately before use, the stacking gel was added and the sample wells formed as follows: stacking gel solution (10ml per cassette) was prepared by mixing 0.16 vol. stock acrylamide (29.8g acrylamide, 0.2g bis-acrylamide to 100 ml of distilled water) 0.25 vol. of stacking gel buffer (0.5M Tris-HCl, pH6.8, 0.4% w/v SDS) and 0.59 volume of distilled water. The mixture was degassed and 0.001 vol. TEMED and 0.01 vol. 10% solution Ammonium persulphate were added.

Quickly, after mixing, a little of the solution was used to

wash the surface of the resolving gel, and the remainder was used to fill the cassette having placed the well-former (comb) in position. A drop of X4 diluted stacking gel buffer was layered onto the gel surface at each air/gel interface. Once the stacking gel has polymerised (30-45 min) the comb was carefully removed and the sample wells were washed with electrode buffer. The spacer forming the base of the cassette was removed and the cassette was placed in the electrophoresis apparatus. Electrode buffer (25mM Tris-HCl, 192mM glycine and 0.1% w/v SDS) were added to the lower and upper tanks. 10µl of 0.001% Bromphenol blue were added to 30µl thawed cell lysate, protein standards were run on the same gel. After loading the samples, electrophoresis was carried out (upper tank as the cathode) at 10mA for 30 min, and subsequently at 20mA until the tracking dye reached the end of the gel.

Staining and de-staining

The slab gels were stained in 0.25% (w/v) Coomassie Brilliant Blue R (1.25g stain dissolved in 227ml methanol, 46ml glacial acetic acid was added and made up to 500ml with distilled water, then filtered). Destaining was done in methanol-acetic acid solution (50ml methanol, 75ml glacial acetic acid made up to 1000ml with distilled water) when protein bands were clearly visible, the gel was photographed.

TWO DIMENSIONAL ELECTROPHORESIS (IEF/SDS)

(developed from O'Farrel, 1975)

Growth and labelling of protein

Cells were grown in low sulphate medium in the presence of $^{35}SO_4^{2-}$ for 14-16h. At a density of 10^7 cells ml^{-1}, while still growing exponentially, the cells were transferred into sporulation medium free of labelled precursors.

Extraction of protein for 2D gel electrophoresis

Samples (10^8 cells) were removed either as they were transferred to sporulation medium (oh sporulation) or at intervals following the transfer. They were pelleted and washed with buffer A (5mM $MgCl_2$, 10mM Tris HCl, pH 7.4) at 0^oC (3000 x g, 5 min). The cell pellet was resuspended in 400µl fresh buffer A (containing pencreatic RNase, 50µg ml^{-1} (EC.3.1.4.22) and 2mM phenylmethylsulphonyl fluoride (PMSF) at 0^oC, and an equal volume of acid-washed 40 mesh glass beads was added. After 2 x 2 min bursts with a vibromix to break the cells, 50µl of buffer A, containing DNase, 50µg ml^{-1} (EC.3.1.4.6) was added and the mixture allowed to stand for 10 min at 0^oC. The sample was then freeze-dried and stored until required. The freeze dried sample was taken up in 400µl of urea sample buffer (9M urea) and after thorough mixing insoluble debris and glass beads were removed by centrifugation (3000 x g, 1 min). 15µl of the supernatant (from 300µl), containing 3-4 x 10^5 cpm as trichloroacetic acid precipitable material, were subjected to isoelectric-focussing.

First dimensional separation

Isoelectric focussing (IEF) tube gels were prepared as follows:

Chromic acid cleaned tubes (Siliconised with 2% Dichlorodimethylsilane in $CHCl_3$) 13cm long with internal diameter 2mm and external diameter 7mm were sealed with parafilm at one end. Stood upright, they were filled (11.0cm) with the following solution: 0.54g Urea, 0.2ml Acrylamide solution (19.67g acrylamide, 0.33g Bis-acrylamide made up to 100ml with distilled water) 0.2ml NP40 (10% [w/v] in distilled water) and 0.05ml Ampholines (pH 3.5-10; 40% solution) made up to 1ml with distilled water. After through mixing polymerisation was initiated with per ml: 1µl TEMED and 2µl Ammonium persulphate (10% w/v solution). The gel surface was overlayed initially with a little distilled water and subsequently after polymerisation with 8M urea. Throughout polymerisation the temperature was maintained above 25°C.

After 60 min polymerisation the tubes were loaded into the gel apparatus. 10µl of 9M urea was loaded onto gel surface and tube filled with degassed 0.1M NaOH as was the top electrode tank (-ve). The bottom electrode tank (+ve) was filled with 0.01M phosphoric acid (H_3PO_4). The apparatus was connected to the power supply and the tubes pre-focussed at initially, 200V and a constant current of 0.3 - 0.5mA/ tube for 1.5 - 2h, during which time the voltage increased to about 400V. After the pre-focussing the tops of the gels were washed with fresh NaOH. Tubes were loaded with 10-20µl of sample which was overlayed with 10µl 8M urea and then fresh 0.1M NaOH. The upper tank was filled with fresh 0.1M NaOH and voltage applied (400V) for a further 19-20h. Temperature was maintained at 25°C throughout.

After IEF, the gels were removed from the tubes (one end

labelled with a piece of cotton) and stood in two washes (1 each) of dialysis buffer (62.5mM Tris-HCl, pH6.8, 2.3% w/v SDS, 5% v/v β-Mercaptoethanol, 10% Glycerol before freezing for storage.

Second dimensional separation

Glass plates (16 x 25cm) with attached perspex levels and supporting plates were washed thoroughly with detergent "Decon" and then alcohol. A cassette was constructed with perspex spacers using a little vaseline on either side of the perspex side spacers and along the bottom edge of the bottom spacer. As little vaseline as possible was allowed to enter the cassette.

The following gel solutions were made:

	15%	5%
Resolving gel buffer (1.5M Tris-HCl pH6.8, 0.4% w/v SDS)	7.5ml	6.25ml
Acrylamide solution (29.8g Acrylamide, 0.2g Bis-acrylamide made to to 100ml with distilled water).	15.0ml	4.16ml
Glycerol	6.0ml	
Water	1.44ml	14.50ml
	30	25

Both solutions were thoroughly mixed and degassed. To 5ml of 15% solution was added 5µl TEMED and 50µl Ammonium persulphate (10% w/v solution), and the mixture was then pipetted into the cassette and overlayed with water-saturated butanol. This produced a sealing gel.

After polymerisation the butanol was washed off and the cassette dried. The remaining aliquots of 15 and 5% solutions were loaded into a gradient maker. (The 15% solution in the mixing chamber). To each chamber was added 30µl TEMED and 30µl Ammonium persulphate (10% solution),50µl of persulphate was added to the 15% solution. After mixing of each solution they were run into the cassette over 15-20 minutes to within 10mm of base of perspex levels. Again the surface of the gel was overlayed with water-saturated butanol. Polymerisation was allowed to proceed overnight.

Immediately before use the butanol was washed away and the cassette was filled almost to the top of the 'V' with the following mixture:

Stacking gel buffer (0.5MTris-HCl pH6.8, 0.4% SDS)	2.5ml
Acrylamide solution (29.8g Acrylamide, 0.2g Bis-acrylamide to 100ml of d.H_2O)	1.5ml
Water	6.0ml

The mixture was degassed before adding 10µl TEMED, and 30µl Ammonium Persulphate (10% w/v solution), poured on top of the gradient gel to form the stacking gel. Whilst the stacking gel was polymerising (30-45 min) the 1D gel was thawed and washed thoroughly with electrode buffer (25mM Tris-HCl, 192mM glycine, 0.1% w/v SDS). Once the stacking gel has polymerised the surface was washed with electrode buffer and the 1D tube gel laid along it. Care was taken to prevent localised stretching of the tube gel. It was

sealed in position with molten 1% w/v agar in electrode buffer containing 0.001% w/v Bromophenol Blue.

The bottom spacer of the cassette was removed and the casette was placed into the apparatus, sealing it in position with vaseline and clamps. Upper and lower tanks were loaded with electrode buffer and connected to a power supply (upper tank - ve). Bubbles trapped in the bottom of the cassette were removed. Constant current was applied at 10mA (30 min) and 20mA for 5-6h.
Once the dye front reached the bottom of the cassette the gel was carefully removed and placed in 10% w/v acetic acid.

Autofluorography of gels (Bonner & Laskey, 1974)

Gels were fixed for at least 2h in 10% w/v acetic acid which was then replaced by 1l dimethyl sulphoxide (DMSO) (wash 1). After standing for 1h this DMSO (reusable 5-6 times) was replaced by a further litre of DMSO (wash 2). Again after another hour this DMSO was removed (reusable 5-6 times as wash 2 and then 2-3 times as wash 1) and the gel immersed in 500ml 20% w/v PPO (biphenyloxalzole) in DMSO for another hour. The dehydrated and PPO impregnated gel was soaked in distilled water to precipitate the PPO. Finally the gel was dried. Dried gels were clipped to a sheet of X-Ray film (Kodak XH1), placed in a metal cassette lined with foam sheet to hold the gel and film firmly. The cassette was wrapped in black plastic bags and placed in a -80^{o}C freezer for the required time.

Exposed films were developed as follows; 4 min in 20% (v/v) Kodak DX80 Developer, 2 min Water wash and 4 min in Kodak FX40

Fixer. The films were thoroughly washed in cold water followed by air-drying. Developed films were carefully examined on a light box.

pH and Molecular Weight Estimation

The pH gradient in the first dimension was estimated by cutting six duplicate isoelectric-focussed tube gels into 5mm slices. Each slice was immersed in 2ml degassed distilled water for 10 min and the pH was measured. Plots of pH versus slice number enable the pH scale to be estimated for the composite diagrams.

Molecular weights of polypeptides seen in the second dimension were estimated by comparison with standard proteins, including bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobulin and lysozyme, separated on a number of gels. In these instances the gels were stained with Coomassie Briliant Blue R (2.5% w/v) and then destained.

Mutagenesis

N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) (according to the method of Dawes et al., 1977).

Cultures were grown to exponential phase in YEPA medium. At a density of 10^7 cells ml^{-1} the culture was harvested, (sporulating cultures were harvested at intervals during the process. Concentration of cells harvested was kept similar to vegetative cultures) washed rapidly with an equal volume of potassium acetate buffer (0.2M; pH 5.5) and resuspended in the same buffer containing NTG (concentration as required). After 10 min at $30^{o}C$, the cells were

harvested by centrifugation and washed twice with acetate buffer containing sodium thiosulphate (1mg ml^{-1}).

Mutagenesis on solid medium was done by placing a minute crystal of NTG in the middle of YEPD plate spread with a lawn of the strain to be tested (the plate was allowed to dry before NTG was added).

UV radiation

Cultures were diluted and plated on YEPD plates and allowed to dry for at least 2h at 30°C, before exposure to UV radiation (1.05J.s^{-1} m^{-2}) for various times. Irradiated plates were incubated in the dark at 30°C.

Mutant selection

Revertants for auxotrophic markers were selected on complete medium lacking the auxotrophic requirements. Isolation of sporulation mutants was done either by microscopic examination or by the method of Dawes & Hardie (1974). Organisms in liquid culture were centrifuged and resuspended in the same volume of 0.2M potassium acetate buffer (pH 5.5). An equal volume of diethyl ether was added to the sample in a 1oz vial and the mixture was emulsified for an appropriate time at ambient temperature. The two phases were then allowed to separate and the lower aqueous layer removed by pipette. For cultures sporulated on plates ether treatment was carried out by replica plating onto YEPD in glass petri dishes and exposing these to ether vapour for 90 min at 30°C in a sealed vessel followed by incubation to detect survivors.

Genetic analysis

The standard techniques of yeast genetic analysis were employed (Mortimer & Hawthorne, 1969).

Crosses were carried out by mixing strains with different genetic markers on YEPD agar plates and selecting for diploids (or tetraploids) on minimal medium. Homothallic diploids were sporulated before mating. Diploids were sporulated on potassium acetate agar plates, a sample of asci was treated with glusulase enzyme (10% solution in 0·2M citrate, 0.1M phosphate buffer pH6) to digest the ascospore wall and tetrads were dissected on agar slabs.

Random spore analysis was done by treating samples of asci with diethyl ether (see above) to kill vegetative cells, then treating with glusulase to free the ascospores. After sonication and appropriate dilution, the sample was plated on YEPD plates.

DNA assay

DNA was assayed according to the method of Stewart (1975). Extraction of DNA : 4ml of cells (2×10^8 cells) were harvested and washed with sterile distilled water, resuspended in 2ml ice cold distilled water and 2ml of 0.5M perchloric acid ($HClO_4$) was added. Samples were incubated for 15 min on ice with occasional shaking. It was then centrifuged, the supernatant was discarded and the process was repeated. The cells were resuspended in 2ml of 0.5M$HClO_4$ and heated for 20 min at 70°C, with shaking at intervals. The extraction was repeated with 1ml of 0.5M $HClO_4$ and supernatants were pooled. DNA assay : 2ml of the supernatant was mixed with 2ml of diphenylamine

reagent (1.5 w/v diphenylamine in glacial acetic acid with sulphuric acid added to a final concentration of 1.5% (v/v), 0.5ml of acetaldehyde solution [16 mg ml^{-1} in distilled water] was added to 100ml solution immediately before use). The mixture was incubated overnight at 30°C in the dark. Absorbance was read at 600nm against a reagent blank.

Nuclear staining

33258 Hoechst (Fabwerke Hoechst AG, Frankfurt):

Staining yeast nuclei with 33258 Hoescht was done according to the method of Lemke et al., (1978). Freshly harvested yeast cells were fixed in absolute isopropanol for 30 min, washed in distilled water and resuspended in 10ml of water containing 50µg ml^{-1} 33258 Hoeschst. Staining time varies from 3h for vegetative cells to 16h for cells undergoing sporulation. All cells were destained in 95% ethanol for 30 min. Stained cells were mounted for examination in 65% w/v solution of sucrose. A Leitz Orthoplan microscope equipped for incident-light fluorescence with a Ploem vertical illuminator was used.

Giemsa stain : This was done according to a modified method of Robinow (1961). A loop of cell suspension was spread thinly on a grease-free glass side, allowed to dry then fixed in Carnoy fixative (3 parts alcohol and one part glacial acetic acid) for 10 min. The slide was dipped in 70% ethanol to remove fixative, followed by a 5 min wash in distilled water. The cells were then hydrolysed in 1NHCl maintained at 60°±0.5°C for 15 min (30 min for sporulating cells).

The slide was rinsed in cold tap water and stained for 15 to 60 min in Giemsa stain solution (4% v/v Giemsa in 0.1M phosphate buffer pH 6.8).

Preparation of thin sections and staining of yeast cells for electron microscopic examination

Samples were taken from vegetative and sporulating cultures (at 2h intervals for sporulating cultures) and washed in distilled water. Part of the sample was fixed in potassium permanganate (1% w/v) solution for 4h (vegetative cells) or 5h (sporulating cells). The other part was fixed in buffered glutaraldehyde (3% w/v glutaraldehyde in phosphate buffer pH7.2) for 24h and post fixed in a buffered 2% w/v osmium tetroxide solution for 1h. Both samples were washed in phosphate bufer, resuspended in 2% liquid agar. When set, the agar was cut in $1mm^3$ blocks and dehydrated in a graded ethanol series (30% to absolute alcohol) followed by two 10 min changes in propylene oxide, then transferred into 50% propylene oxide to 50% TAAB 812 resin mixture. The cells were infiltrated with TAAB 812 resin mixture for 15h, then 4h in fresh resin mixture. After blocking the resin was polymerised for 12h at $60^{o}C$.

Sections were cut on an OMU3 microtome with glass knife, collected and mounted on copper grids (400 mesh) and stained with saturated aqueous uranyl acetate and lead citrate (Reynolds, 1963). Grids were examined using an EAI EM6 electron microscope.

Transformation of E. coli strain HB101 (modified from Lederberg & Cohen, 1974)

A fresh overnight culture was diluted 1.50 into L-broth, and grown at 37°C with shaking to a turbidity of 0.55-0.65 (650 nm) cells were chilled on ice for 20 min, pelleted and resuspended in half the initial volume of ice-cold 0.1M Magnesium Chloride, then immediately repelleted at 4°C, resuspended in 1/15 - 1/20th of the initial volume of ice-cold 0.1M $CaCl_2$ and left on ice for 30 min.

Plasmid DNA was diluted in SSC:$CaCl_2$ in an Eppendorf tube (1× SSC [0.15M NaCl, 0.015M Sodium Citrate pH7], 0.1M $CaCl_2$; ratio 3:4) to a final concentration of 1μg ml^{-1}. 0.2ml of treated cells were added to DNA solution and left on ice for 30 min. It was given a heat shock for 2 min at 40°C, then immediately chilled on ice for 5 min. 1ml of pre-warmed L-broth was added to the DNA and recipient mixture, incubated at 37°C with shaking for 1h. The cells were diluted and plated on L-Agar with ampicillin added (100 μg ml^{-1}) and incubated over night at 37°C.

Preparation of plasmid DNA from E. coli HB101 transformants (Modified method of Birnboim, 1979)

Transformed strain HB101 was grown in L-broth to a turbidity of 0.6 (650nm) and treated overnight with chloramphenicol at 170μg ml^{-1} to amplify the plasmid. 0.5ml of cell culture was centrifuged for 15 s , then resuspended in 100μl lysis solution (freshly prepared and kept at 0°C : lysozyme, 2mg ml^{-1}, 25mM Tris - HCl pH 8.0, 100mM Ethylenediaminetetra acetic acid [disodium salt]

EDTA; 50mM glucose) at 0°C for 30 min. The mixture was thoroughly mixed, 200µl alkaline SDS solution (0.2N NaOH, 1% w/v SDS) was added and left at 0°C for 5 min followed by 150µl high salt solution (3M Sodium acetate, pH 4.8, 300µg ml^{-1} carrier tRNA added shortly before use) and incubated at 0°C for 60 min., with occasional mixing.

The mixture was centrifuged for 5 min at room temperature, 400µl supernatants was collected avoiding any floating precipitate and 1 ml of ethanol was added to the supernatant. After storage at -20°C for 30 min, the mixture was centrifuged for 2 min. The supernatant was aspirated carefully and the pellet dried in a nitrogen gas stream. The pellet was redissolved in 100µl dilute sodium acetate (0.1M, pH6.0), and 200µl cold ethanol was added and stored at -20°C for 10 min. It was centrifuged for 2 min and the supernatant was discarded. The final pellet was the plasmid DNA. It was dissolved in 75µl 1 x SSC buffer and analysed by electrophoresis on agarose gel. Plasmid DNA used in transformation experiments was purified by phenol/chloroform extraction and treatment with RNase.

Agarose gel analysis of plasmid DNA (Modified method of Sharp et al., 1973)

`Glass plates used were thoroughly washed, rinsed in distilled water and dried. Cassettes were prepared as for one-dimensional PAGE but thicker Perspex spacers (4mm) were used. One of the plate was coated with 0.1% w/v agarose to prevent the gel sliding during electrophoresis.

Agarose gel (1.25g agarose in 125ml gel buffer [0.04M Tris-HCl, 0.02M Sodium acetate, 0.01M EDTA]) was dissolved by refluxing and cooled to 50°C. It was poured into the cassette with slot former at the top and sealed at the bottom with parafilm. A small amount of agarose was poured and allowed to set at the bottom, before the gel was poured. When the slab gel was set the slot former was removed and the slots half filled with buffer. The cassette was placed in electrophoresis apparatus and samples (with 10µl 20% Ficoll and 0.025% Brophenol blue) were loaded using a micropipette. The gel was run from cathod to anode at 15mA (running buffer was the same as the gel buffer).

Horizontal gel was prepared on a Perpex plate with two side pieces attached and two detachable end pieces. The apparatus was placed on a level surface with the slot former held in position with plasticene so that the teeth were about 1mm clear of the base plate. Agarose solution (prepared as above) was poured directly into the apparatus. When set the plate was rested between two buffer tanks and wicks made of Whatman No. 1 chromatography paper were used to connect the gel to the running buffer. After loading the sample mixture (sample + 10µl 20% Ficoll and 0.025% Bromophenol blue) the gel was run at 40mA.

Agarose gels were stained by soaking in a solution of Ethidium bromide (0.5µg ml^{-1}) for 1h, destaining in distilled water for another 1h and photographed with UV illumination from beneath the gel.

Preparation of yeast protoplasts and transformation to LEU^+ phenotype (method of Beggs, 1978)

Cultures of S. cerevisiae to be transformed were grown in YEPD, and collected at 10^8 cells ml^{-1}. Cells were washed in sterile distilled water, concentrated threefold in 1.2M sorbitol, 25mM EDTA, 50mM dithiothreitol (pH8) and incubated at 30°C for 10 min with gentle shaking. Cells were washed twice by centrifugation and resuspension in the original culture volume of cold sterile 1.2M sorbitol. Protoplasts were obtained by concentrating the cells three-fold in 1.2M sorbitol, 0.01 M EDTA, 0.1M sodium citrate (pH 5.8) containing filter-sterilised Helicase (lyophilised snail gut extracts (Industrie, Biologique, Francaise) at 2mg ml^{-1}, incubated at 30°C with gentle shaking for 1h. Protoplasts were washed by centrifugation and resuspension in the original culture volume of 1.2M sorbitol three times to remove nuclease activities present in the snail gut extract, and resuspended in 1.2M sorbitol containing 10mM $CaCl_2$ to give approximately 10^{10} protoplasts ml^{-1}. DNA was mixed with 0.1ml protoplasts to give 20µg ml^{-1}. Protoplasts and DNA were left at room temperature for 15 min and then diluted with 10 volumes of 20% (w/v) polyethyleneglycol 4000, 10mM $CaCl_2$, 10mM Tris-HCl (pH 7.5). After 20 min protoplasts were pelleted and resuspended in 100ul 1.2M sorbitol, 10mM $CaCl_2$, 20µg ml^{-1} leucine plus 50µl YEPD containing 1.2M sorbitol and incubated at 30°C for 20 min. Samples were diluted in 1.2M sorbitol and plated by mixing in 7ml minimal medium containing 1.2M sorbitol and 3% (w/v) agar at 45°C and pouring onto minimal medium plates containing 1.2M sorbitol, 2% agar. Dilutions were plated on supplemented (20µg leucine ml^{-1})

minimal plates to measure the efficiency of protoplast regeneration. All plates were incubated at 30°C for 5 to 7 days.

To test for the presence of transforming plasmid in yeast (Method of Beggs, 1978).

Single colony of transformants were streaked on YEPD (1 x 2 cm) and incubated overnight at 30°C. These streaks of cells were resuspended in 0.5ml 1.2M sorbitol, 25mM EDTA, 0.86M β-mercaptoethanol, pH 8.0, left at room temperature for 20 min, then centrifuged. The pelletted cells were resuspended in 0.5ml 1.2M sorbitol, 0.1M sodium citrate, 10mM EDTA pH 5.8 containing Helicase snail gut extract at 2mg ml^{-1} and incubated at 30°C for 1.5h. Protoplasts were washed twice by centrifugation and resuspended in 1.5ml 1.2M sorbitol and finally resuspended in 200µl 10mM Tris-HCl, 1mM EDTA, 1% SDS pH8. Protoplasts were lysed by incubation at 60°C for 20 min and debris was pelletted. Lysate (40µl) was mixed with 10µl 20% Ficoll, 0.025% Bromophenol blue and electrophoresed through 1% agarose slab gel.

RESULTS AND DISCUSSION

SECTION I

HETEROGENEITY OF CULTURE POPULATIONS WITH RESPECT TO STAGE OF SPORULATION AND TO UPTAKE OF AMINO ACIDS

Recent techniques such as those developed by Fast (1973) and Roth & Halvorson (1969) have made it possible to induce sporulation with a fairly high degree of synchrony. This is adequate for most purposes in the studies of development of ascospore formation in Saccharomyces cerevisiae. There are however, situations in which the residual asynchrony and the presence of non-sporulating cells in the cultures can distort the results obtained; this is particularly the case when sequential changes are under study and pulse labelling techniques are being used, as in experiments to demonstrate specific protein changes during yeast sporulation which had failed to show changes. Therefore, there is a need to consider that pulse labelling may not be an appropriate technique.

It has also been reported that uptake of labelled precursors was markedly reduced during sporulation and this was considered to be due to the increase in pH which is necessary for successful sporulation (Mills, 1972). This pH change may well alter the permeability of the cell membrane.

In order to study this effect further, we developed a technique employing Urografin gradients to resolve sporulating populations into fractions containing cells at different stages of the processes of meiosis and sporulation. This method was used to illustrate the heterogeneity of cultures during sporulation and to show marked differences that occur between cells in the various stages of

sporulation with respect to the incorporation of labelled amino acids. Colloidal silica (Percoll) gradients were also tried as a meansof separating sporulating populations. The results were quantitatively similar to that of Urografin gradients.

Genotypes of strain 60 x 61

Strain 60 x 61 $\left(\frac{a\ HO\ ade\ 2\text{-}40\ his\ 4\text{-}239\ ARG\ 4\text{-}1\ MET13\ can\ 1\ CYH2}{\alpha\ HO\ ade\ 2\text{-}119\ his\ 4\text{-}166\ arg\ 4\text{-}1\ met13\ CAN\ 1\ cyh\ 2}\right)$

was constructed from a variety of strains including some supplied by Dr. F. Zimmerman, Darmstadt, F.R.G. and the Yeast Genetic Stock Culture Collection, Berkeley, USA. The HO gene confers homothallism. The ade 2-40 and ade 2-119 alleles complement and the parent diploid is adenine-independent with white colonies. However once meiosis and segregation of the nuclei has occurred, the haploid progeny require adenine and give colonies that are either red (ade 2-40) or pink (ade 2-119, a leaky mutation). Thus a cell committed to completing meiosis can be scored directly after mating on any medium. Cells committed to recombinatio were scored by testing for loss of the histidine requirement due to recombination between the non-complementing alleles of the his 4 gene complex. The genes can 1 and cyh 2 confer recessive resistances to L-canavanine sulphate (80µg ml^{-1}) and to cycloheximide (5µg ml^{-1}), respectively. These genes are both present in the heterozygous state and the appearances of resistant organisms can also be used as marker events during sporulation.

Separation of sporulating cultures on Urografin gradients

Initial experiments using linear Urografin gradients covering various density ranges indicated that sporulating yeast populations could be resolved into a number of bands by centrifugation, whereas under the same conditions vegetative populations formed a single main band. Best results were obtained by layering 0.8 - 1ml of culture

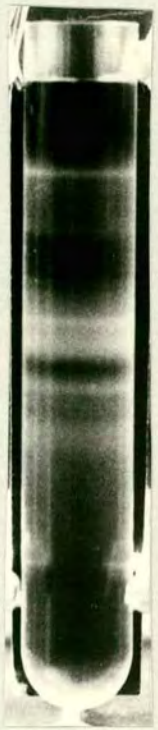
over a very shallow gradient (density 1.15 to 1.20 g cm^{-3}) in long tubes (97 mm, 16.5ml capacity) and centrifuging at low speed (4500g) for 10-15 min.

The centrifugation time affected the pattern of bands seen after centrifugation. Periods of 10-15 minutes were not long enough for cells to attain their eventual equilibrium position. Longer centrifugation times tended, however, to draw the bands together, and if enough time was allowed, at equilibrium, a diffuse broad band formed. These changes were complex, and hence both the speed and time of centrifugation were standardised in subsequent experiments. The changes may have been due to the cells taking time to reach the position expected from their initial density, and also to the cells gradually changing in density due to loss of water by osmosis, or to slow penetration of Urografin. The better separations may therefore be due to a combination of cell density, cell size and cell permeability to Urografin. By using very shallow gradients separations such as the one illustrated in Figure 1.1a were obtained.

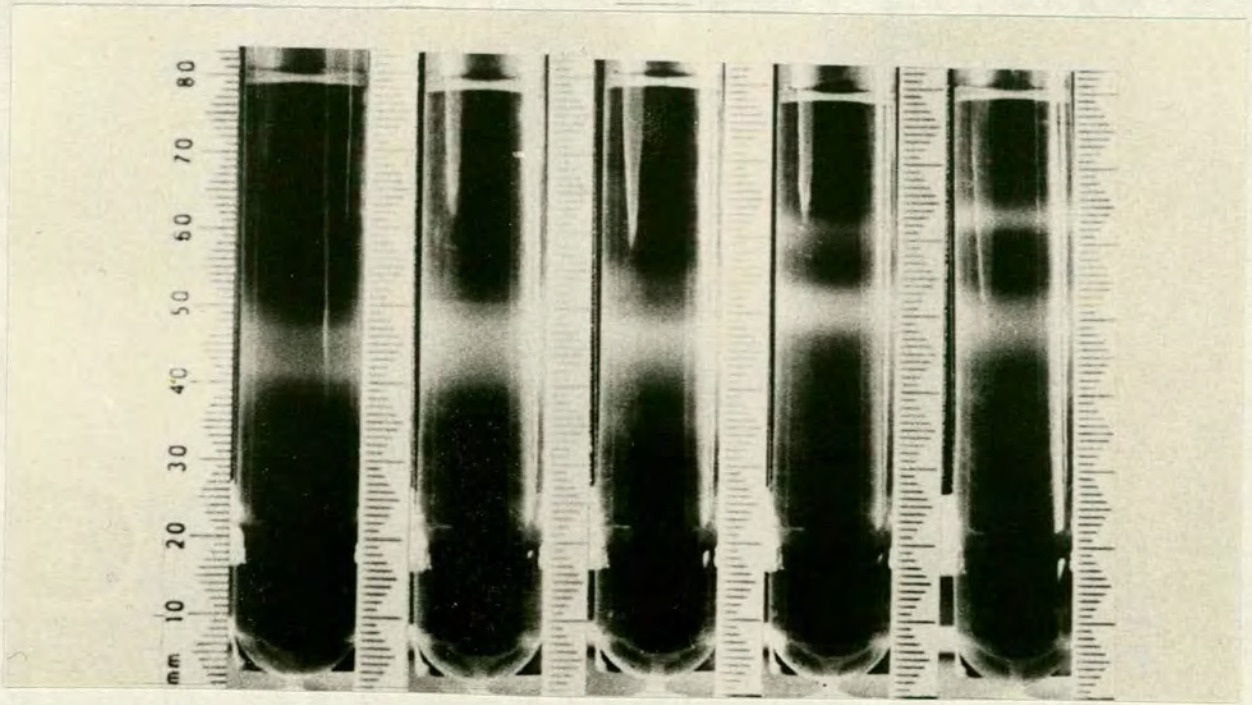
Resolution of cultures at different times after induction of sporulation

Figure 1.1b shows the changes in the pattern of cell banding when cultures were separated at 5 h intervals throughout sporulation. At each time, a 1ml sample was layered on a Urografin gradient with density range from 1.13 to 1.22g cm^{-3} and this was centrifuged for 15 min.

Samples taken at 5 hours into sporulation, and later, exhibited discrete bands, indicating that distinct physiological stages



(a)



(b)

Figure 1.1 : Separation of sporulation cultures into subpopulations. (a) The pattern of cell banding observed on centrifugation of a 20h sporulation culture through a linear gradient of Urografin (Centrifugation time, 15 min; density range, 1.13 to 1.20 g cm^{-3}). (b) The effect of sporulation time: samples taken at the times indicated after resuspension in sporulation medium were separated on Urografin gradients (centrifugation for 15 min; density range, 1.13 to 1.22g cm^{-3}).

are reached by the cells placed under sporulation conditions, and that the transition of cells from one state to another is fairly rapid, otherwise the cells would have been distributed much more broadly. As sporulation proceeded, significant changes occurred leading to an increased complexity of the banding pattern obtained at later sporulation times. Lower density fractions appeared, as shown by Figure1.1b.

Heterogeneity of sporulating cultures

Fractions from cultures at various stages of sporulation separated on Urografin gradients were collected and analysed to see if they contained cells at different stages of meiosis and sporulation They were microscopically observed and analysed for events characteristic of sporulation. These included : commitment to intragenic recombination (by scoring for intragenic recombination at the his 4 locus); commitment to meiosis (by segregation of the ade 2 gene) and the appearance of recognisable asci. It was obvious that there was a marked increase in the heterogeneity of sporulating cultures with time after resuspension under conditions supporting meiosis and sporulation.

Figure 1.2 shows the data obtained from a 16.5h sporulating culture separated on a very shallow gradient. In strain 60 x 61, tetrads normally began to appear between 15 and 16h after resuspension in sporulation medium, and these reached a maximum of about 70% of the population at 20 to 24h. Most (80%) of the tetrads were four-spored under the conditions used. From Figure1.2 it can be seen that cells in

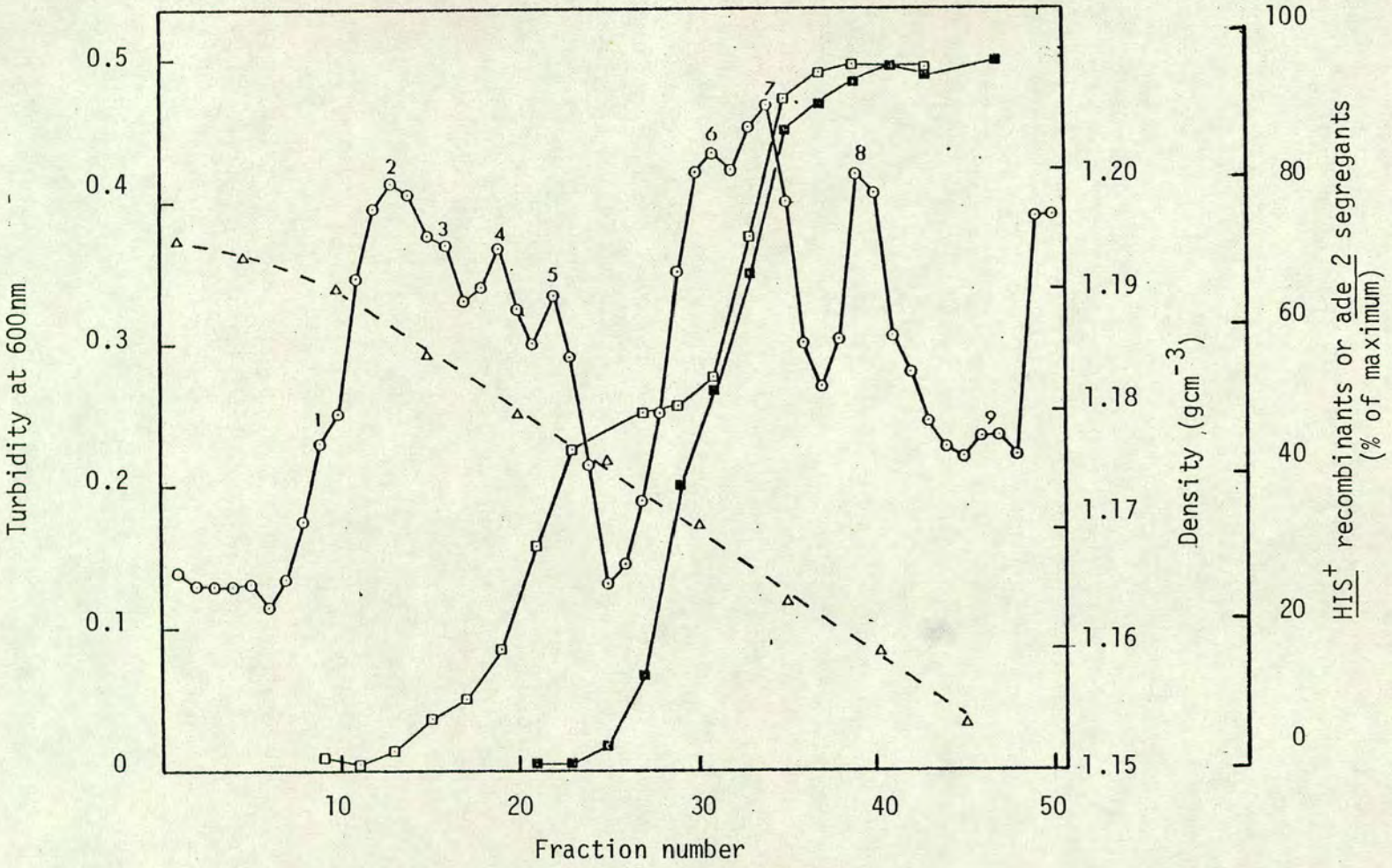


Figure 1.2. Heterogeneity of sporulating cultures. A 16.5h sporulation culture was separated on a linear gradient (1.15 to 1.195 g.cm^{-3}). The tube was pierced, fractions were collected from the bottom of the gradient, and the following were estimated: turbidity at 600nm (o); commitment to recombination at the his 4 locus (□); commitment to meiosis (■); density of Urografin (△). Data were plotted normalising the maximum extents of recombination and meiosis to a value of 100%; this corresponded to a value of 6.1×10^3 histidine-independent recombinants ml^{-1}, and to 35% red-sectored colonies. Peaks are numbered 1 to 9.

the lower, denser part of the gradient were uncommitted to either recombination or meiosis, and that only those in the upper bands (number 5 to 8 in Figures 1-2) showed any signs of segregation of the ade 2 markers. Histidine-independent recombinants were detected in band 5 as well as bands 6 to 9, but the maximum frequency was not seen until band 7. The majority of cells in bands 1 to 5 were unbudded, and no asci were seen on microscopic examination. In band 6 no mature asci were found, but the populations contained a high proportion of cells in which the first signs of ascus formation were just evident. Band 7 contained approximately 10% mature tetrads; the remaining cells were immature asci similar to those seen in band 6. Fraction 8 contained more than 60% tetrads and cells in the upper region of the gradient were almost all mature asci.

These data indicated that after 16.5h in sporulation conditions the cells were broadly distributed throughout the various stages of sporulation. About half of the population was not yet committed to meiotic recombination (at the his 4 locus) although several percent had completed recombination, meiosis and the formation of mature spores.

Uptake and incorporation of labelled amino acids

Pulse-labelling techniques have considerable use in tracing the fate of metabolites, or in studying the synthesis of particular molecules, but the results of these need to be viewed with caution when using heterogenous populations. As indicated above, sporulating populations consist of cells at various stages and clearly any measurements made

on an unresolved sample taken at any particular time from such a population could give a misleading impression of events taking place at a particular stage of sporulation. It has also been reported that sporulating cultures incorporate precursors of macromolecules at a much lower rate than vegetatively growing cells (Esposito et al., 1970; Magee & Hopper, 1974; Mills, 1972). Thus, it was decided to examine the uptake and incorporation of two amino acids, L-phenylalanine and L-arginine, by different sub populations in sporulating cultures. In these experiments two methods were used to label the cultures. The first involved the direct addition of the labelled amino acids to a sporulating culture, and after 10 mins incubation it was centrifuged through an appropriate Urografin gradient. The second method was that of Mills (1972) in which cells from sporulating cultures were harvested by rapid centrifugation and were resuspended in potassium acetate (0.2M) buffered to pH 6 prior to the addition of the labelled amino acid.

The results from both methods were qualitatively similar Figure 1.3 shows the extent to which the different cell types took up the incorporated L-[^{14}C] arginine when a 21h culture containing 20% asci was pulse-labelled for 10min in buffer at pH6 prior to separation on a Urografin gradient. In this experiment total radioactivity, and that precipitated by cold 10% (W/V) TCA were estimated. The distribution of total activity and of TCA-insoluble activity was similar except at the top of the gradient where unincorporated label remained during centrifugation. A very marked difference was seen between different cell fractions in the extent of uptake and incorporation of label relative to the concentration of cells in each fraction. Figure 1-4 shows the incorporation by a

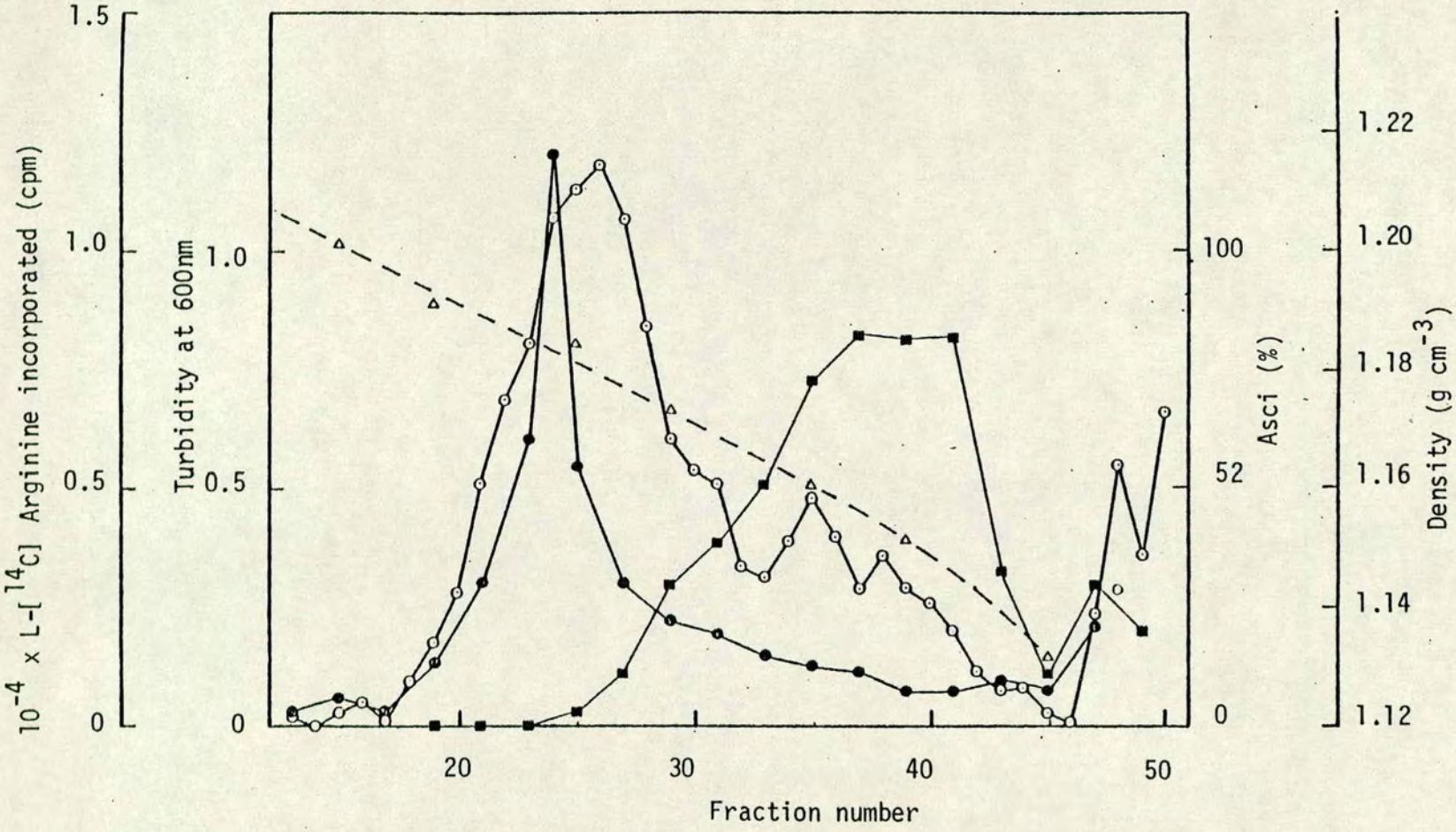


Figure 1.3. Incorporation of L-[^{14}C] arginine by subpopulations in a 21h sporulation culture. After pulse-labelling with arginine for 10 min at pH6 the culture was separated on a linear gradient (1.13 to 1.20 g cm^{-3}). Fractions were collected and assayed for: turbidity at 600nm (o); arginine incorporated into TCA precipitate (●); percent asci (■); and density of Urografin(△).

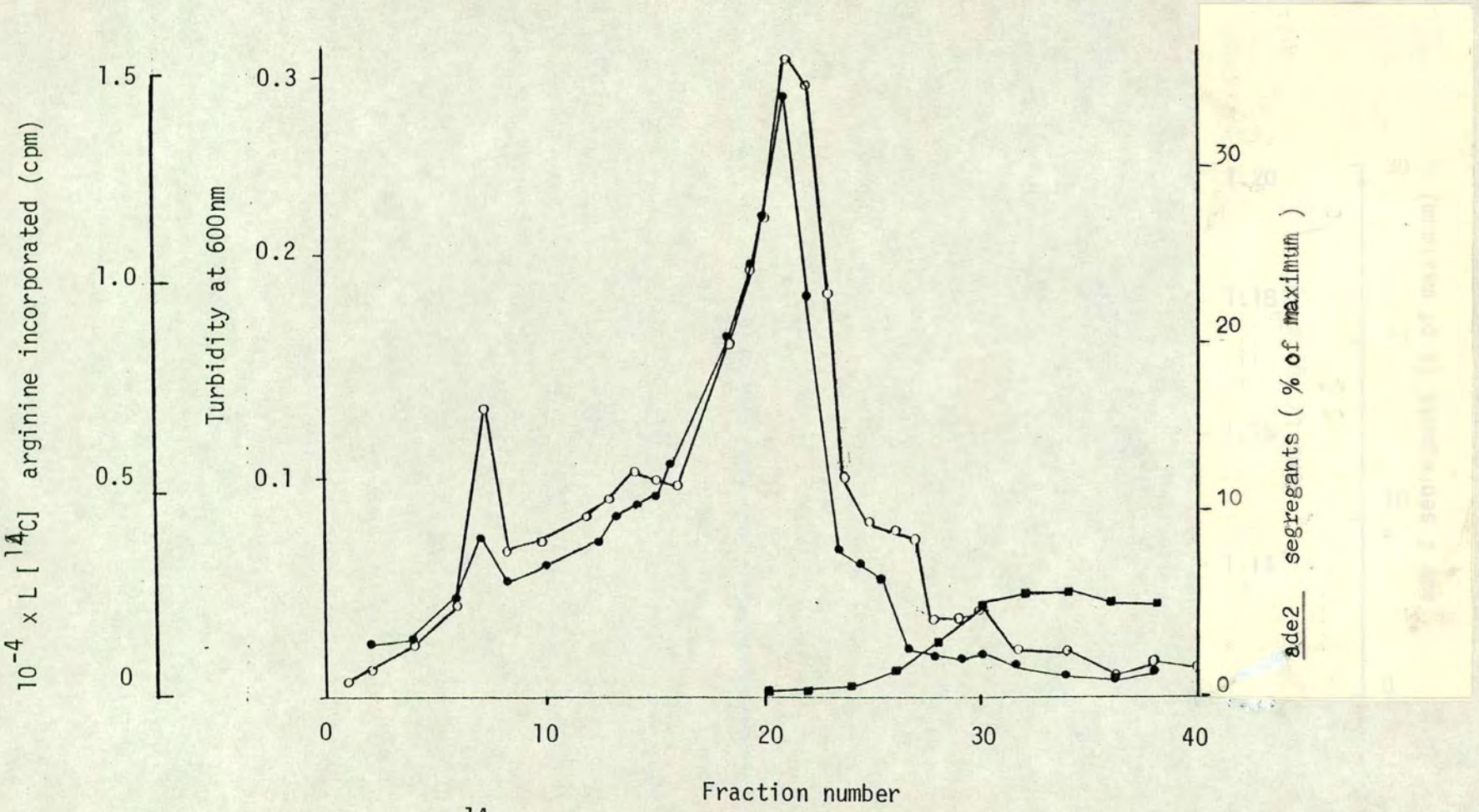


Figure 1.4 : Incorporation of 1-[^{14}C] arginine by subpopulation in a 12h sporulating culture. Pulse-labelling was done without adjusting the pH, then separated on a linear gradient (1.13 to 1.20 g cm^{-3}). Fractions were collected and assayed for: turbidity at 600nm (o); arginine incorporated into TCA precipitate (●); commitment to meiotic segregation (■).

12h sporulating culture to which the L-[^{14}C] arginine was added directly. It was clear that most of the label was taken up by cells which were not committed to meiosis.

The experiment was repeated using a different amino acid, L-phenylalanine to see if the effect was restricted to arginine, and also using an earlier culture (16h) under improved separation conditions. The result given in Figure 1·5 confirmed the finding that the majority of labelled precursor taken up during a 10 min pulse was found in those cells that had not yet begun to undergo meiosis or sporulation or that were at a very early stage in meiosis.

Comparative studies with Percoll gradient

Percoll is composed of colloidal silica coated with polyvinyl-pyrrolidone (PVP) which renders it non toxic to living cells. It is also impermeable to biological membranes resulting in no change of buoyant density of particles during centrifugation. Thus this material seemed ideal for the formation of gradients to separate population of living cells. As recommended by the manufacturer, several Percoll gradients balanced in salts and sucrose solutions were tested for separating sporulating populations of Saccharomyces cerevisiae. The best separation was obtained by balancing 9 parts (V/V) Percoll with 1 part (V/V) 10 x concentrated salt solution (10 x 9% NaCl) and progressively diluted* with the salt solution (9% NaCl). Figure 1.6 shows the separation of a population after 18h resuspension in sporulation medium. Microscopic examination indicated that the population was separated according to the stages reached by cells in

* i.e. making a gradient with salt solution

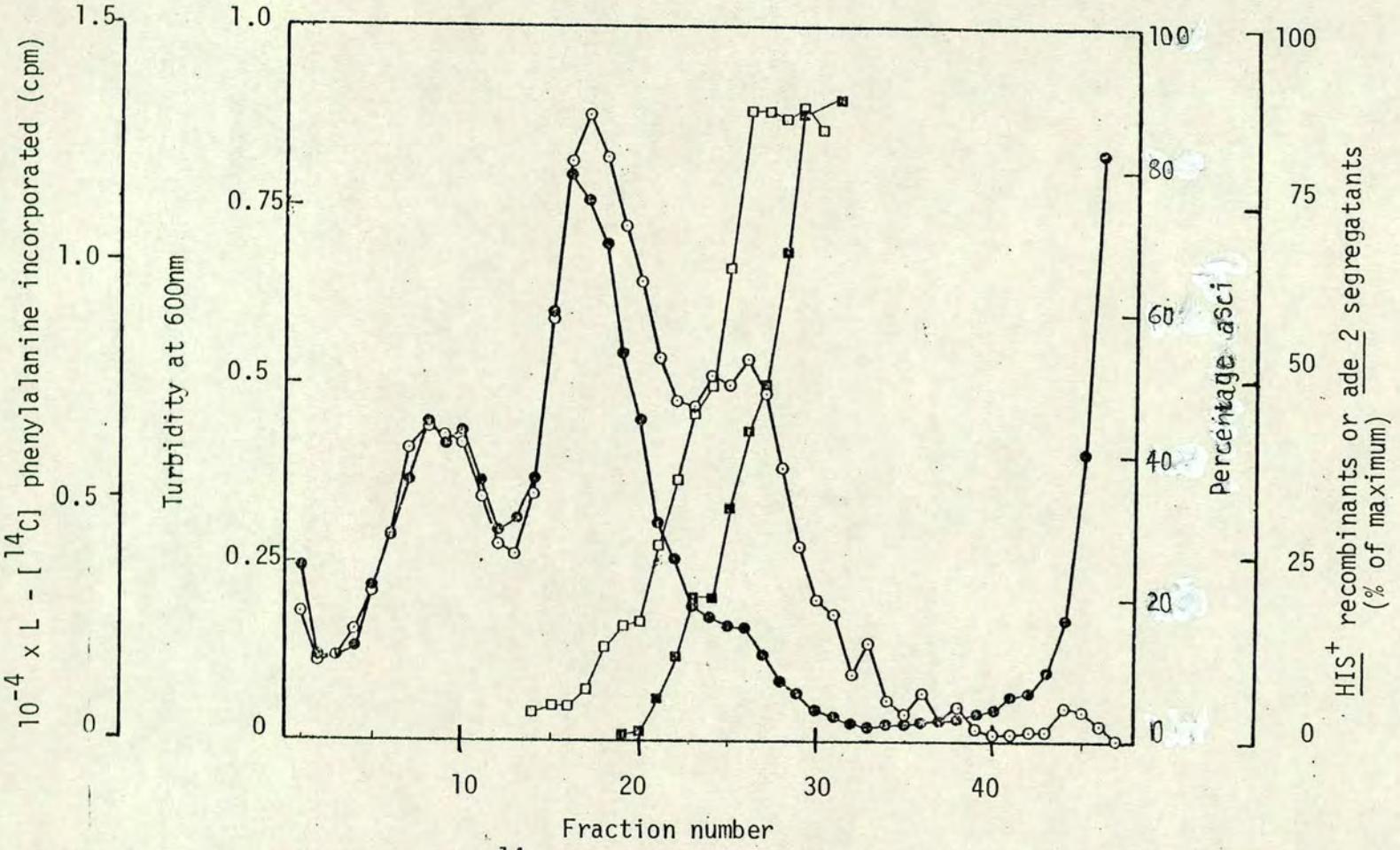


Figure 1.5 : Incorporation of L-[^{14}C] phenylalanine by cells in a 16h sporulating culture. After pulse-labelling for 10 min at pH6 the culture was separated on a linear gradient (1.13 to 1.20 g cm^{-3}). Fractions were collected and assayed for turbidity at 600nm (o) L-(^{14}C) phenylalanine incorporation into TCA precipitate (●), commitment to recombination at the his 4 locus (□) commitment to meiosis (■).

Figure 1.6. Heterogeneity of sporulating cultures. A 18h sporulating culture was separated on Percoll-sodium chloride gradient. The tube was pierced, fractions were collected from the bottom of the gradient and the following were estimated, turbidity at 600nm (o), percentage asci (■), density of Percoll (△).

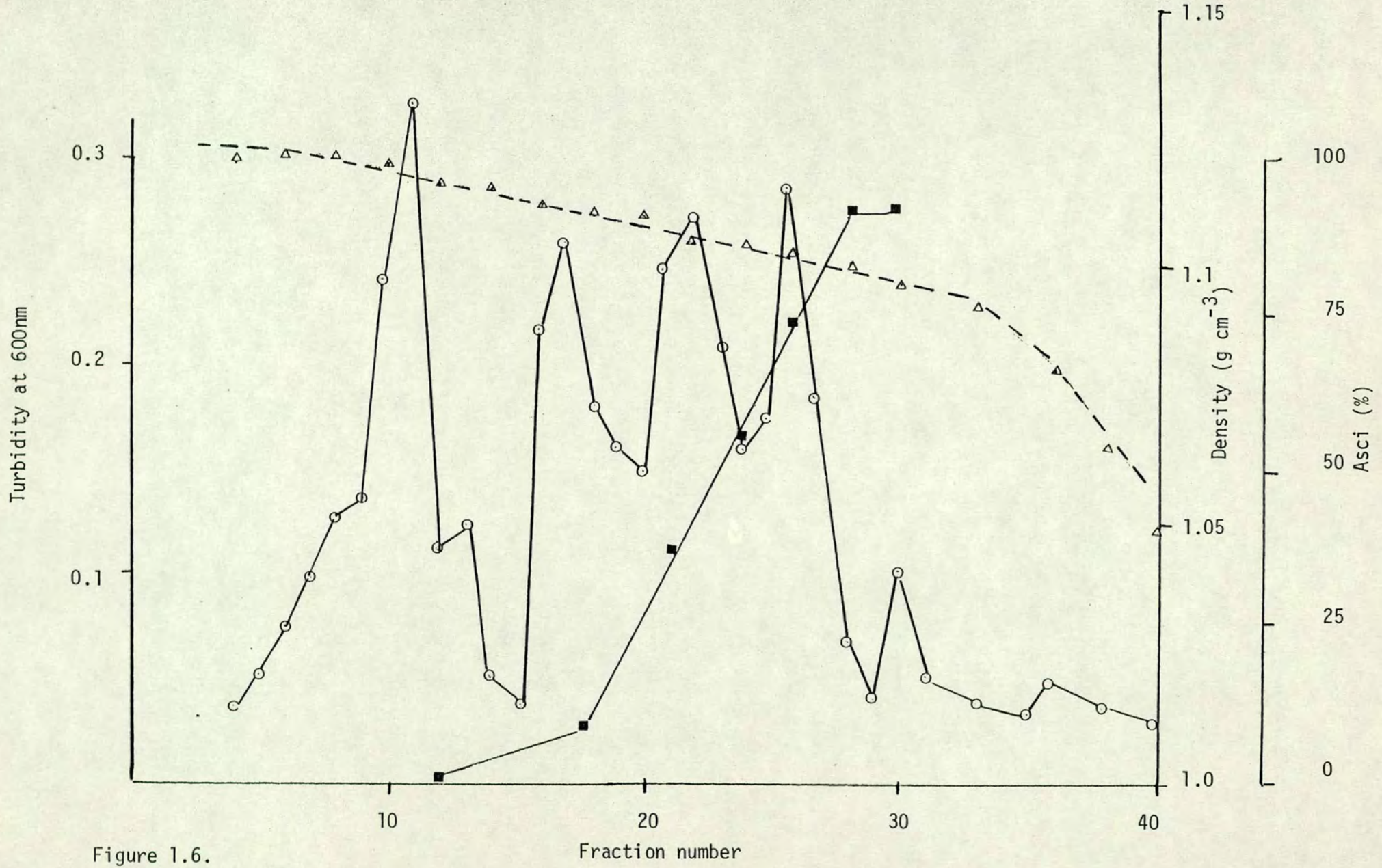

Turbidity at 600nm
0.3
0.2
0.1
Density (g cm^{-3})
1.15
1.1
1.05
1.0
Asci (%)
100
75
50
25
0
10
20
30
40
Fraction number

Figure 1.6.

meiosis and sporulation: the vegetative cells were most dense and the spores formed a distinct band at the top of the gradient.

Uptake and incorporation of labelled amino acids

Using Percoll gradients of similar ranges, the uptake and incorporation of labelled amino acids were examined. Both methods of labelling were employed. There were no apparent differences between the results obtained by either method. However the separation of the population seemed to be affected by resuspending cells in potassium acetate solution (0.2M) at pH 6 for the 10 min labelling period. The bands formed were more diffuse than those labelled directly. Figure 1.7 presents results obtained by this method. As found using Urografin gradients most of the L-[^{14}C] arginine was incorporated into cells that had not reached the point of commitment to meiosis and spore formation. The distinct bands formed as shown in Figure 1.6 were very diffuse in Figure 1.7.

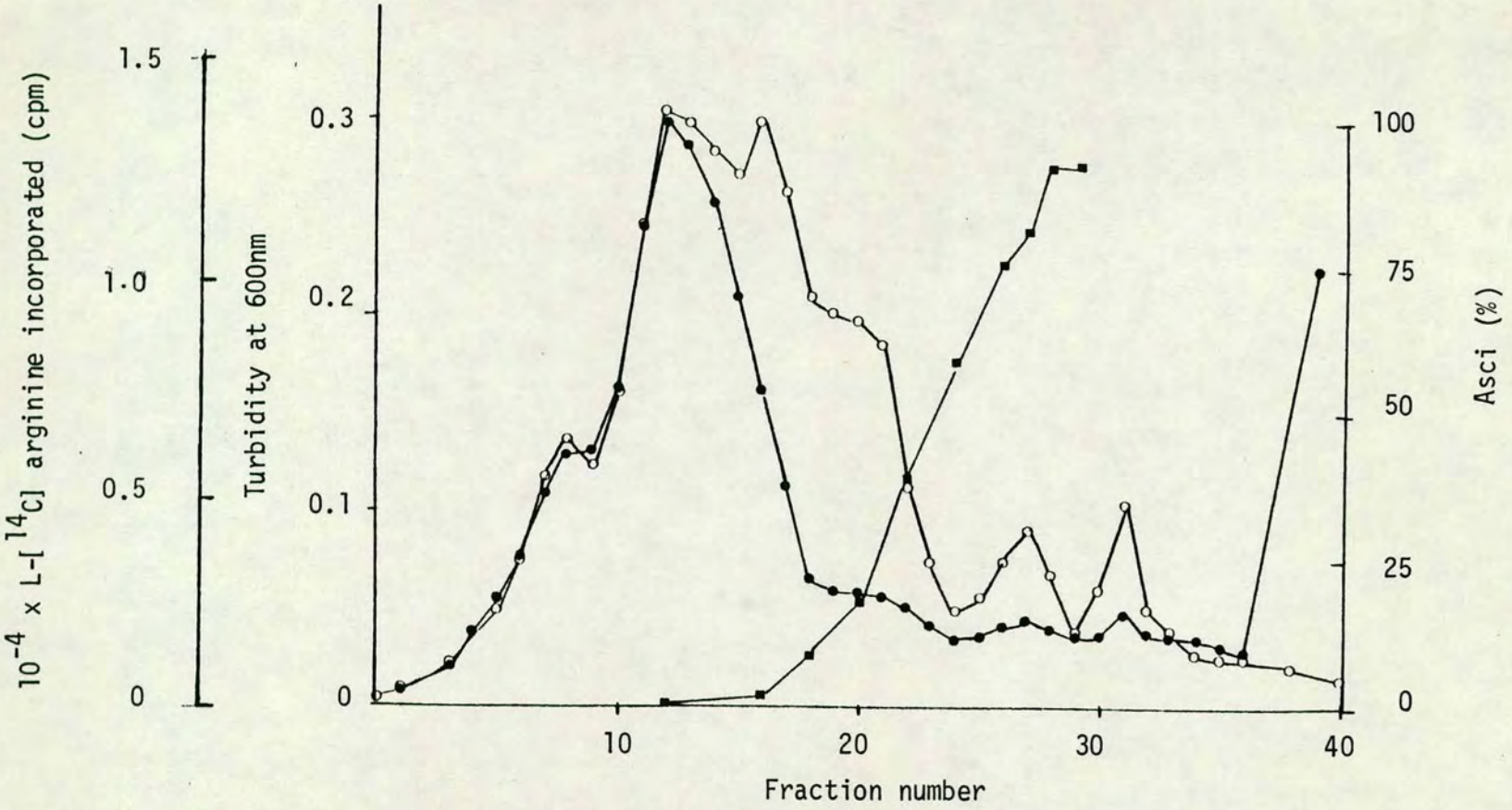


Figure 1.7 : Incorporation of L-[^{14}C] arginine by subpopulations in a 20h sporulating culture. After pulse labelling with arginine for 10 min at pH6 the culture was separated on a Percoll gradient. Fractions were collected and assayed for turbidity (o), arginine incorporated into TCA precipitate (●), percentage asci (■).

Discussion

The separation of sporulating cultures on Urografin gradients enables resolution of a single culture into fractions containing cells at certain physiological stages of meiosis or sporulation. For example, it was possible to obtain a fraction of cells that were committed to meiotic recombination but not yet committed to meiotic segregation. With care and using very shallow gradients up to seven or eight fractions can be obtained. From the results presented, it is clear that as cells progress further into sporulation they band at positions of lower density on the Urografin gradient. The transition from one state to another seemed to be fairly rapid. Since the separation method does not depend on density alone, their physiological basis is not clear. The changes in lipid synthesis (Henry & Halvorson, 1973) that accompany sporulation would lead to a decrease in cell density and could contribute to the effect. Other processes which may be important include the breakdown of glycogen (Kane & Roth, 1974) and the synthesis of the spore coat structures which may alter the density of the ascus and the permeability of parts of it. One of the main shifts in band position of the cells seems to begin when the outline of developing ascospores can be discerned under phase contrast, corresponding to the changes from band 5 to band 6 in Figures 1.2.

Separation of a population into cells at the various stages of meiosis and sporulation is not limited to Urografin gradients only, Percoll balanced in 9% sodium chloride solution gave comparable results.

Several problems were encountered using this method. First, rather large amounts of Urografin were needed to separate relatively small samples, and unless the Urografin were to be recovered the technique could prove expensive if large amounts of material were needed for subsequent analysis. Different strains of Saccharomyces cerevisiae also required preliminary characterisation to establish the useful density range for the gradients. A problem that arose on several occasions, and for which no clear explanation has been found, was the flocculation or aggregation of cells in the Urografin. A number of variables were tested, including the use of various salts, buffers of different pH, and lower sample loadings. None of these affected the aggregation markedly, although lowering the sample loading helped a little. In practice the problem was remedied by recloning the yeast strain used.

Flocculation was not apparent on Percoll gradient but separation seemed to be affected by pH change of the resuspension buffer of the culture. Percoll has a pH of 8.8 and the pH of a sporulating culture is about 9. There must be slight changes in the cell properties when they were exposed to the 10 min. labelling in potassium acetate (0.2M) at pH 6 which made the bands diffuse. A note should also be made about the recovery of the bands from the centrifuge tubes after centrifugation. The data presented here were obtained by pumping off the gradient from below via a peristatic pump. It has been recently been found that this leads to considerable broadening of the bands, and better results can be achieved by avoiding the use of pumps on the outlet side of the tube.

Despite these limitations, the technique has considerable potential for a number of biochemical studies of meiosis and sporulation in yeast, since it enables resolution of cells at different physiological stages from a single culture. Most of all, it highlighted the problem of labelling sporulating cultures, as most of the labelled precursors were preferentially taken up by cells which were not yet committed to meiosis and sporulation. Results of some biochemical and genetic studies of such populations should, therefore, be interpreted with caution.

SECTION II

SPECIFIC PROTEIN CHANGES DURING SPORULATION AND THE TIMING OF THEIR APPEARANCES

It has been shown that protein synthesis is an absolute requirement for successful sporulation in Saccharomyces cerevisiae. Inhibition of synthesis prevents any further development (Esposito et al., 1969; Magee & Hopper, 1974). Genetic data have also been taken to indicate that at least 50 loci code for functions that are indispensible during sporulation (Esposito et al., 1972). Despite all these observations attempts to identify sporulation-specific protein changes by pulse-labelling sporulating cultures with amino acids and subsequently examining their polypeptide complement by the one-dimensional (Hopper et al., 1974) or two-dimensional (Petersen et al., 1979; Trew et al., 1979) polyacrylamide gel electrophoresis (PAGE) have met with little success. Changes were detected in these studies but they also occurred in isogenic non-sporulating cells (such as a/a and α/α diploids) and haploids placed under sporulation conditions.

A sporulating population is very heterogenous in terms of the presence of cells at different stages of the process. Although several techniques were available to synchronise such populations (Fast, 1973; Roth & Halvorson, 1969), these may not be sufficient for detecting stage-dependent changes such as changes in the pattern of gene transcription. Physiological changes also occurred as sporulation proceeded which in turn brought about changes in properties of the cells. As shown in the preliminary studies, there was a tendency for preferential uptake of precursors by cells of early stages which have not yet become committed to meiosis. All these factors could have masked the results

of pulse-labelling studies.

To avoid some of these problems, a different approach of labelling cells for identifying sporulation specific changes was developed by Wright & Dawes (1979). This technique involved labelling vegetative cells by growing them in a galactose minimal medium containing $^{35}SO_4^{2-}$ (galactose allows depression of mitochondria), then suspending them in a sulphur-free sporulation medium. Under these conditions any polypeptide that is either modified, or synthesised de novo during sporulation will appear as a distinct new species on two-dimensional gel electrophoresis. In their initial experiments, several sporulation-specific changes were detected. This chapter reports that by employing a similar technique, further detailed experiments have shown that the changes observed were sequential, each occurring at a characteristic time during sporulation.

The phenotypes of strains JW1 and JW2

Strain JW1 $\frac{a}{\alpha}$ $\frac{ARG\ 4\text{-}17}{arg\ 4\text{-}17}$ $\frac{his\ 5\text{-}2}{his\ 5\text{-}2}$ $\frac{LEU\ 2\text{-}1}{leu\ 2\text{-}1}$ $\frac{lys\ 1\text{-}1}{lys\ 1\text{-}1}$ $\frac{spd1\text{-}1}{spd1\text{-}1}$ $\frac{TRP1\text{-}1}{trp1\text{-}1}$ $\frac{URA3\text{-}1}{ura3\text{-}1}$

was constructed from spores of strains carrying the spd 1 phenotype defined by Dawes (1975). The spd 1 gene ensured not only extensive sporulation but also that sporulation was more synchronous. These two factors are essential to ensure representative results when looking at sequential events during sporulation.

Strain JW2 was derived from strain JW1. The latter was UV irradiated to induce gene conversion at the mating-type locus. In strains obtained with a/a or α/α configuration, sporulation is blocked, thus this provides control strains isogenic to JW1 in all regions other than at the mating-type locus.

Growth and sporulation responses of JW1 and JW2

JW1 showed comparable growth to ordinary a/α diploids on glucose-containing media (Figure 2.1). However due to its derepressed phenotype, it can initiate sporulation in media in which wild type strains are repressed. In media in which glycerol was the carbon substrate, growth was slightly repressed as shown in Figure 2.1, and most of the cells tended to undergo sporulation as soon as possible, probably immediately after the obligatory round of mitosis. Derepression of sporulation usually requires depletion of carbon and nitrogen sources, however, JW1 sporulated as soon as glucose was depleted even when the nitrogen ($NH4^+$) level was still high. Sporulation was also extensive in acetate medium (as is the case with cells growing on

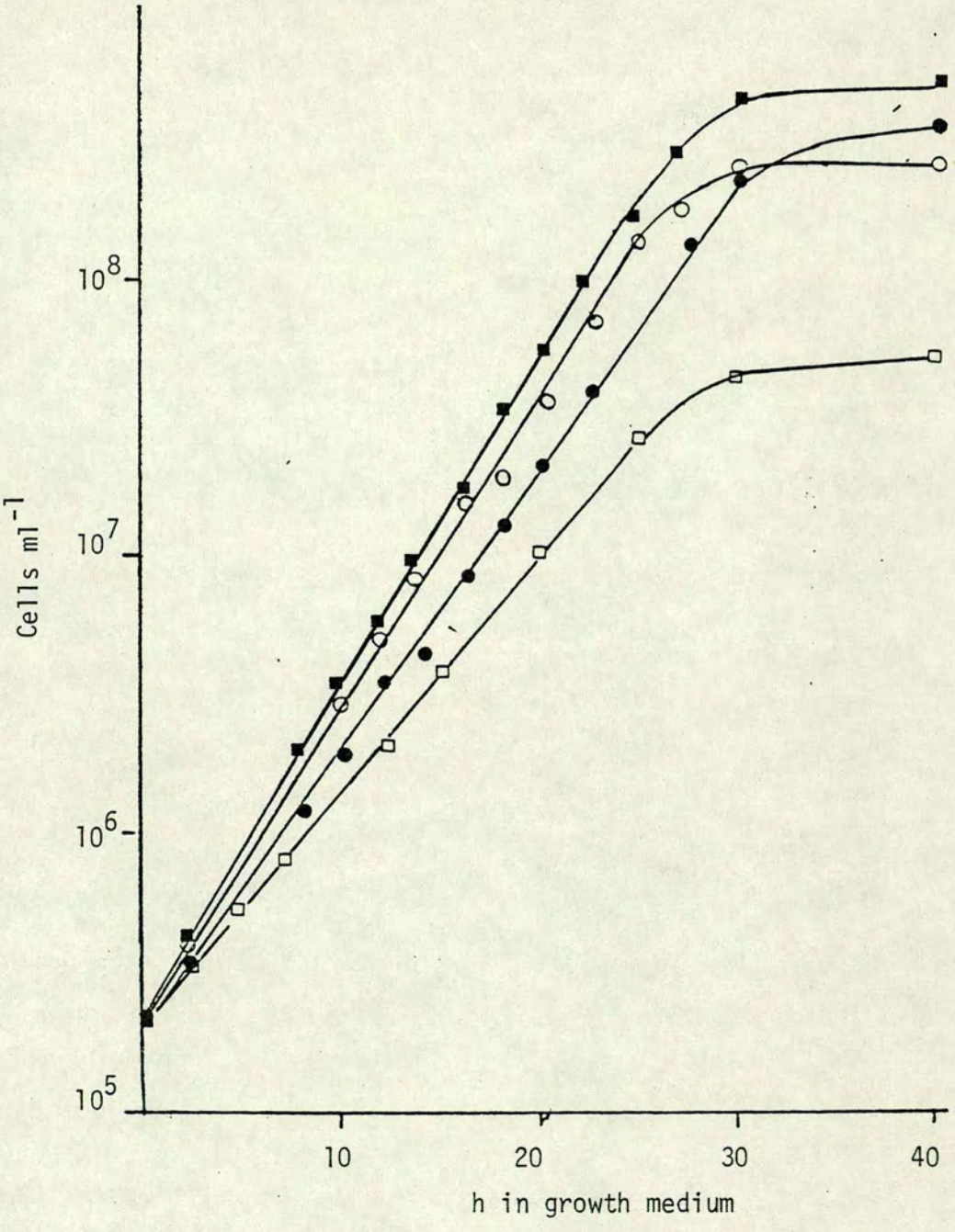


Figure 2.1 : Growth of strain JW1 in medium with different carbon sources: (■) YEPD, (○) YEPA, (●) YEPGAL, (□) YEPG. Cells were grown in liquid medium with shaking at 30°C. Samples were taken at times indicated.

glycerol) this is partly due to derepression of the TCA cycle, which is an absolute requirement for sporulation (Kuenzi et al., 1974). As shown in Figure 2.2, most of the cells were competent to begin sporulation events (in this case, premeiotic DNA replication) immediately when an exponentially growing culture from an acetate medium was resuspended into sporulation medium. The cellular DNA content rose immediately and the first asci were observed ~ 6h after resuspension.

When galactose was used as a carbon source in the presporulation growth medium, strain JW1 behaved in the same way as wild type strains when transferred into sporulation conditions. Figure 2.3 illustrates the timing of sporulation events in strain JW1. Premeiotic DNA synthesis was initiated 3h after resuspension, followed by commit ment to meiotic segregation as determined by the appearance of leu 2 auxotrophs and asci could first be detected after 12 hours. The majority (about 75%) were four-spored, while most of the remainder were three-spored.

Yeast utilises galactose in the same way as glucose through the fermentative pathway, but galactose does not repress the TCA cycle enzymes or other mitochondrial functions required for sporulation. At the same time it does not allow derepressed strains such as JW1 and others (Kinnard & Dawes, 1979) to start sporulation events prematurely in the growth medium.

JW2 showed similar growth kinetics to JW1 in all the media tested but did not show any asci or any earlier events that occurred during meiosis and spore formation when it was resuspended in sporulation

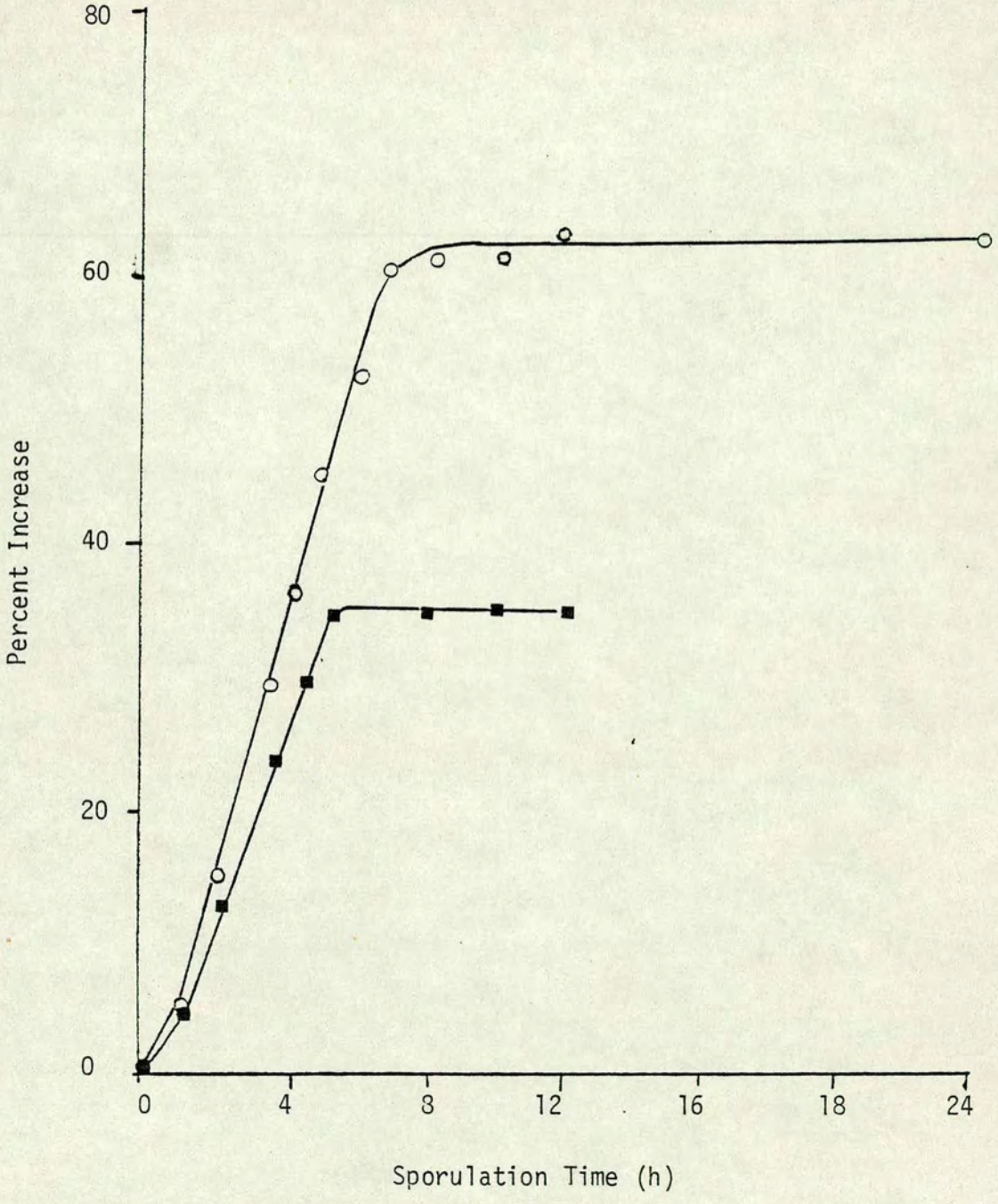


Figure 2.2 : Timing of some sporulation events in strain JW1 pregrown in acetate medium, at logarithmic phase transferred into sporulation medium. (ò) DNA per ml of culture (■) commitment to meiotic segregation, plotted as twice the percentage of ura3 segregants observed. The first ascus was observed after 6h.

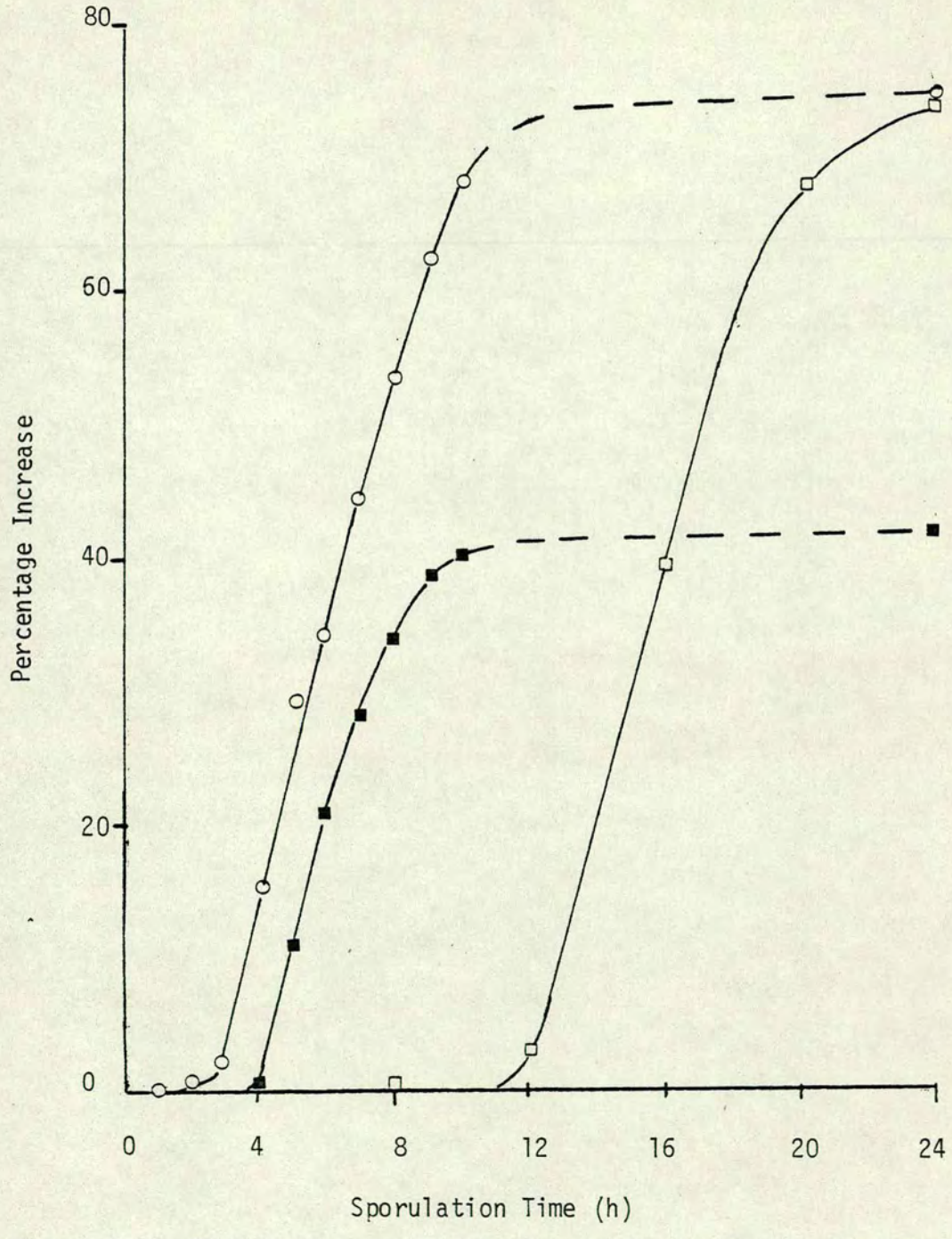


Figure 2.3 : Timing of some sporulation events in strain JW1 pregrown in galactose medium then at logarithmic phase transferred into sporulation medium : (○) DNA per ml of culture, (■) commitment to meiotic segregation, plotted as twice the percentage of leu 2 segregants observed, (□) percentage asci.

medium.

Pregrowth in low S-galactose medium and uptake of $^{35}SO_4^{2-}$

In order to avoid the difficulties arising from differential uptake of labelled precursors (section I) JW1 and JW2 were labelled continuously during the pregrowth period (Wright & Dawes, 1979). Since the label was in the form of soluble sulphate, a low sulphate medium with galactose as carbon source was used. The culture was allowed to grow for about 6-8 generations during this period, the label from the $^{35}SO_4^{2-}$ is extensively incorporated into both vegetative cell proteins and the amino acid pools of the yeast. Thus any sulphur-containing proteins synthesised during the subsequent sporulation of these cells would be labelled with either the free amino acids or those released by the breakdown of labelled protein. Furthermore should sporulation involve the specific modification of pre-existing vegatative polypeptides the new specific forms would also be labelled. Hence by this approach changes during sporulation due to either modification or *de novo* synthesis would be detectable.

The prelabelling resulted in steady-state labelled cells with an uptake of less than 50% of the isotope. The subsequent extraction of polypeptides, allowing for losses due to the counting methods and trichloroacetic acid precipitation gave roughly 50% recovery of this isotope in the 300µl of supernatant taken for analysis. The remainder could be accounted for in unrecovered sample buffer, insoluble cell debris and in material adhering to glass beads.

One-dimensional gel electrophoresis of protein extracts from JW1 and JW2

Protein extracts from sporulating cultures of JW1 and JW2 were prepared at 0, 4, 8 and 24h and subjected to electrophoresis on 7-15% (W/V) gradient polyacrylamide gels containing sodium dodecyl sulphate (SDS). Best results were obtained by staining, since labelled extracts tend to give diffused bands, quantitatively there were no differences between the results of the stained gels and those obtained from the labelled extracts. Figure 2.4 illustrates the results obtained from strain JW1, proteins separated and stained with coomassie blue. JW2 showed exactly the same pattern of polypeptide species. Synthesis of several vegetative proteins ceased during exposure to sporulation conditions, the most obvious of these were seen as two prominent polypeptides of high molecular weight which had disappeared completely after 4h in sporulation medium. A few other bands became more distinct indicating that significant synthesis had taken place. Most of the changes observed occurred fairly early after resuspension into sporulation medium.

The results were similar to those observed in other strains by Hopper et al., (1974) who used pulse-labelling techniques, and thus at the level of resolution achieved by one-dimensional PAGE no sporulation-specific polypeptide changes could be observed. However, this cannot be taken to imply there is no such change, and the high resolution of cellular polypeptides that could be achieved using the two-dimensional technique devised by O'Farrell (1975) was therefore used.

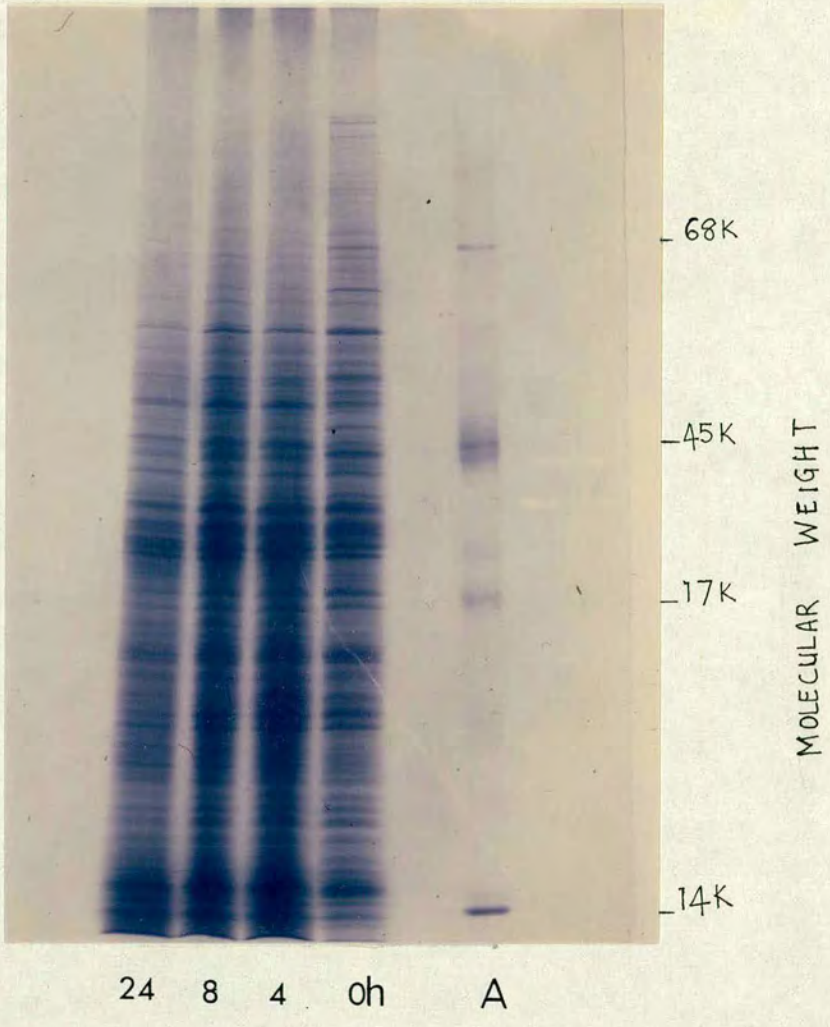


Figure 2.4 : One-dimensional gel-electrophoresis of protein extracts at 0, 4, 8 & 24h during sporulation of JW1 stained with coomassie blue. Lane A are protein standards.

Two-dimensional gel electrophoresis of [^{35}S] - labelled protein extracts from cultures of JW1 and JW2 exposed to sporulation conditions

^{35}S-labelled extracts from JW1 and JW2 prepared at 2h intervals during sporulation were first subjected to isoelectric focussing (pH 3-10) in tube gels of 20% polyacrylamide gel for 18 to 20h, then separated by polyacrylamide gel electrophoresis on 5-15% (W/V) gradients of acrylamide in the presence of sodium dodecyl sulphate (SDS) according to the method of O'Farrell (1975).

Changes occurring during exposure of JW1 and JW2 to sporulation conditions

A composite diagram (Figure 2.7) of readily detectable polypeptides from Saccharomyces cerevisiae was constructed by comparing the autofluorograms obtained by 2-dimensional PAGE of ^{35}S-labelled extracts prepared at 2h intervals throughout sporulation. Each extract was analysed at least in duplicate, and each gel exposed for autoflurography for several different times. As reported by Wright and Dawes (1979), 400 prominent polypeptides were detected. 45 of the total polypeptides showed significant changes during 24h in sporulation medium. The mode of changes can be divided into 3 groups:

Changes specific to sporulating cells

14 new spots (1, 2, 7, 11, 16, 17, 20, 23, 24, 26, 27, 35, 38 and 39) seemed to be apparent in the a/α sporulating cells only. 7 spots (4, 10, 13, 14, 28, 37 and 45) increased in intensity throughout the 24h period after resuspension into sporulation medium. The alterations/synthesis of polypeptides were probably specifically

Figure 2.5 : Autofluorogram of [^{35}S] - labelled proteins from a/α 0h sporulating cells, separated in the first dimension by isoelectric focussing (pH 3-10) and in the second according to molecular weight in a 5-15% polyacrylamide gel slab containing 0.1% SDS. The gel was exposed to X-Ray film for 6d.

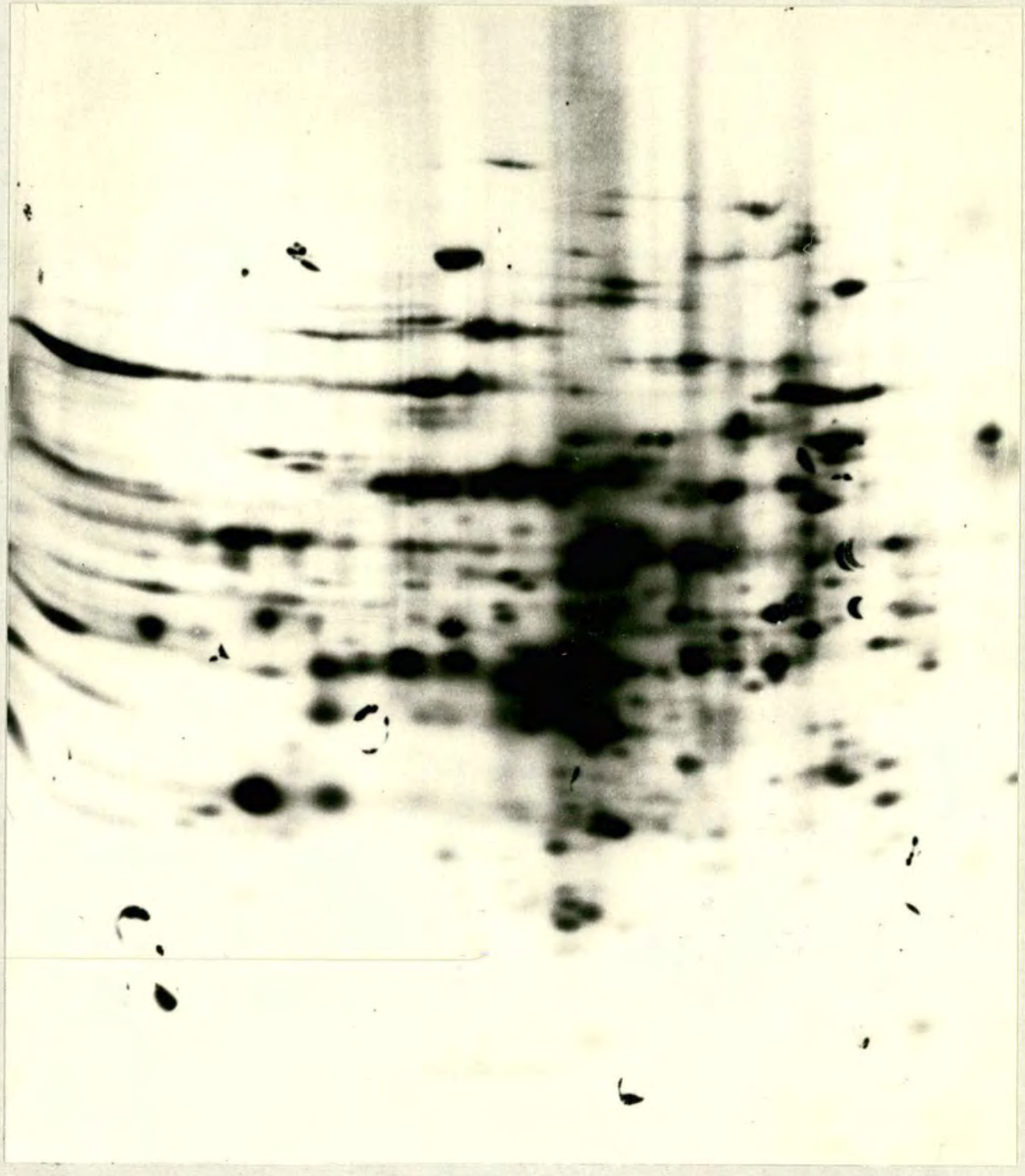


Figure 2.6 : Autofluoro gram of [^{35}S] - labelled proteins from a/α 8h sporulating cells separated in the first dimension by isoelectric focussing (pH 3-10) and in the second according to molecular weight in a 5-15% polyacrylamide gel slab containing 0.1% SDS. The gel was exposed to X-Ray film for 8d.

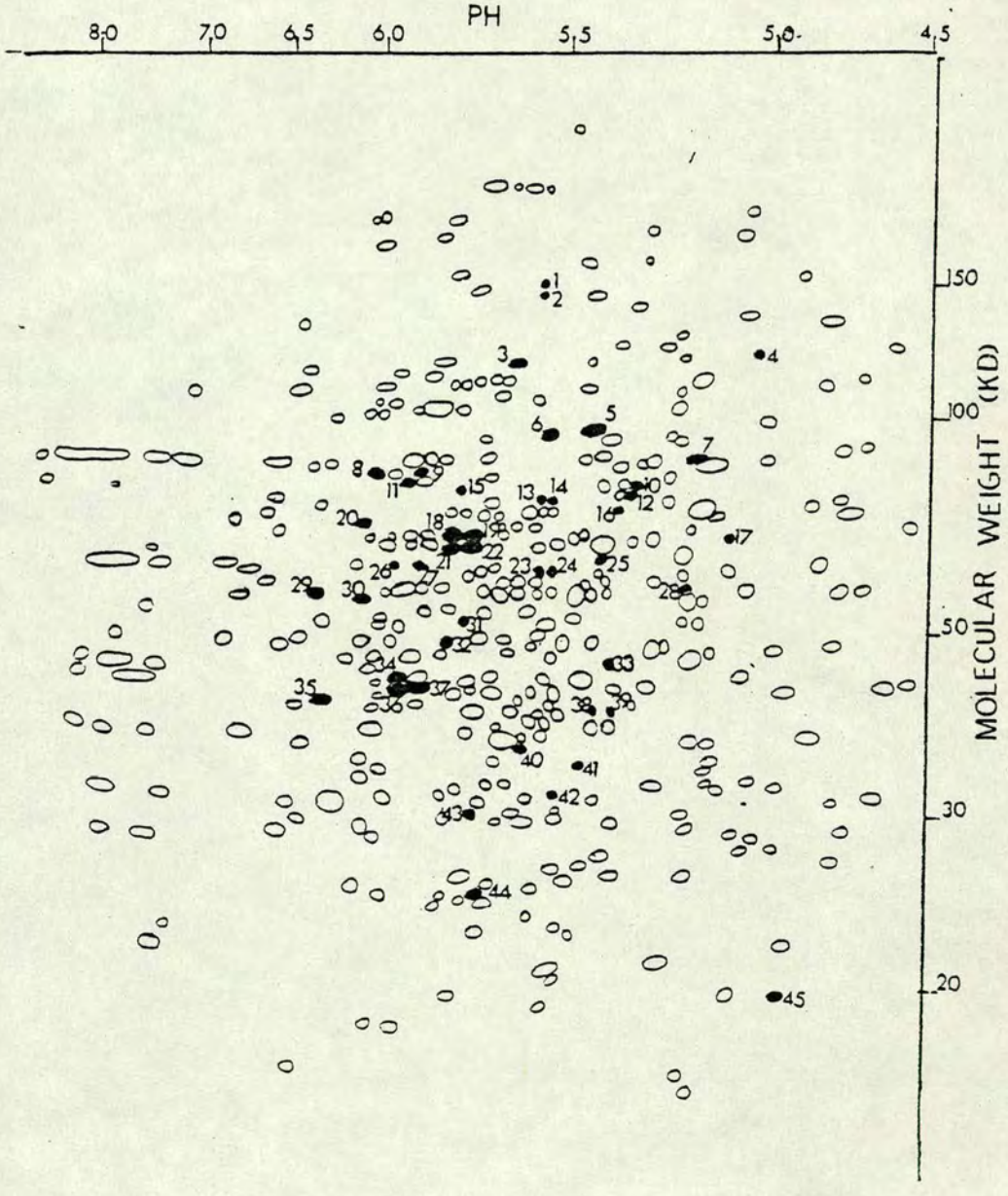


Figure 2.7 : Composite diagram of the most prominant polypeptides, constructed by comparing the autofluorograms obtained by 2-dimensional gel electrophoresis of [^{35}S]- labelled extracts prepared at 2h intervals throughout sporulation. Those polypeptides undergoing changes during sporulation are indicated as blacked-in spots and numbered for reference to Tables 2.1, 4.1 and 4.2.

Table 2.1: The Nature and Timing of Significant Changes in Polypeptides during Sporulation

Nature of Change	Identity of Polypeptide [a] and Time of First Appearance [b] (hr)				
	2	4	6	8	16-24
Sporulation-specific Appearances					
- NEW	7, 17, 20 26, 27, 35	11, 16, 23 38	24, 39		1, 2
- Concentration Increase	4, 13, 14 37	10, 45		28	
Common Appearances					
- NEW	29, 40, 43 44	12	33		
- Concentration Increase	5, 18, 19	3, 31, 32	21, 22, 30 34, 36		
Other Alterations		41	8, 15	9, 42	6, 25

[a] Polypeptides are numbered according to the scheme given in Figure 2.7

[b] Unless otherwise stated in the text the change persists for the remainder of the 24hr period.

associated with the sporulation process.

Changes common to both sporulating and non-sporulating cells

Six polypeptides (12, 29, 33, 40, 43, 44) appeared in both the a/α sporulating and the a/a non-sporulating strains. Eleven more polypeptides showed concentration increases in both strains, as judged by changes in intensity of exposure. These polypeptides were probably involved indirectly in the sporulation process or they were changes brought about by the cells adapting to the change in the nutritional environment.

Other alterations

Seven other polypeptides showed different types of changes from those mentioned above. Spot 42 disappeared completely from a/a cells only, and two more (6 and 25) were reduced in intensity. Another spot (15) disappeared completely and two, 8 and 9 were reduced in intensity in both a/α and a/a cells. Spot 41, although appearing in both a/α and a/a cells, was much more evident in a/α cells.

The above changes occurred in polypeptides from a wide spectrum of molecular weights, in the range from 20,000 to over 150,000 with most from 40,000 to 90,000. Appearances of proteins of high molecular weight would be unlikely to be due to proteolytic changes occurring during extraction. All possible precautions were taken to avoid artifacts of this nature in accordance with the practice of others. Moreover, a mixed cell extraction procedure was used to

eliminate the possibility that changes were due to proteolysis during extraction. Unlabelled sporulating cells were mixed with labelled vegetative cells and extracts were made in the usual way. No significant differences between the labelled polypeptide pattern of the mixture and of the 0h extracts was found.

The nature of timing of the protein changes during sporulation

By examining autofluorograms of extracts prepared at successive two-hourly intervals after transfer of cells to sporulation mediums, individual polypeptides were seen to alter at characteristic times. Figures 2.8 A, B and C illustrate the changes that occurred in polypeptides numbered 34 to 37 on the composite diagram (with molecular weights about 45,000 and PI about 6.0). 34 and 36 were polypeptides showing increasing concentration in both a/α and a/a diploids, 35 was a sporulation-specific polypeptide that appeared early and 37 increased in concentration in a/α sporulating cells only. Figure 2.9 illustrates changes of the polypeptides in a/a nonsporulating cells, in which polypeptide 35 was not detected. Figures 2.10 A, B and C show another region of the autofluorograms in which several other changes can be seen.

The majority of change detected, both sporulating-specific and those common to both cell types occurred within the first six hours in sporulation medium (Table 2.1), at least six hours before the first appearance of asci as determined by light microscopy. Ten of the 21 sporulation-specific changes were detectable after only 2h in sporulation medium and eight of the rest had occurred within a further

Figure 2.8 : The timing of changes in some polypeptides during sporulation
Autofluorograms of the separated [^{35}S] - labelled polypeptides were obtained on cultures that had been pre-labelled with $^{35}SO_4^{2-}$ prior to resuspension in sulphur-free sporulation medium. Samples were taken at (A) 0h, 2h. (B) 4h, 6h. (C) 8h and 24h after resuspension. Only the small region of each autofluorogram including polypeptides 34 to 37 and those with molecular weights about 45,000 and PI 6 is shown.

The gels for Figures 2.8 and 2.9 were exposed to X-Ray films for 7d.

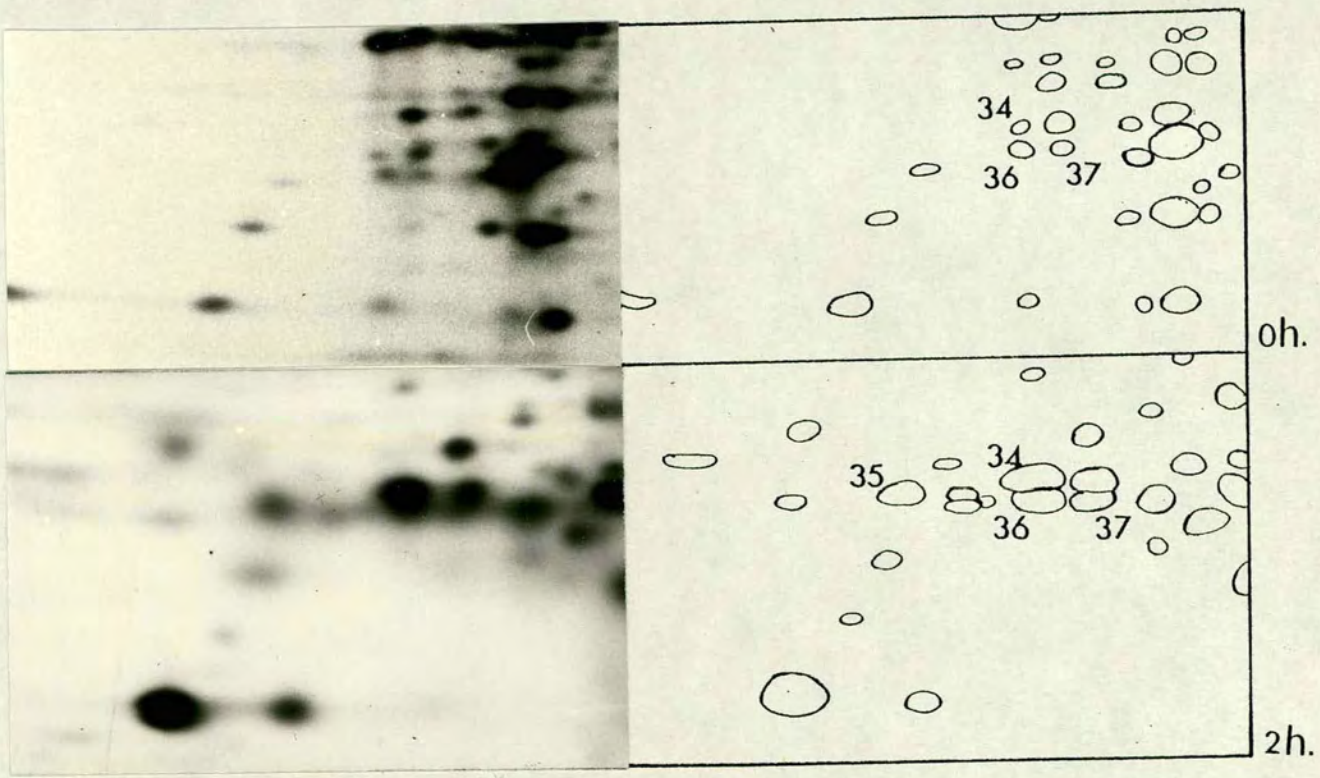


Figure 2.8 A

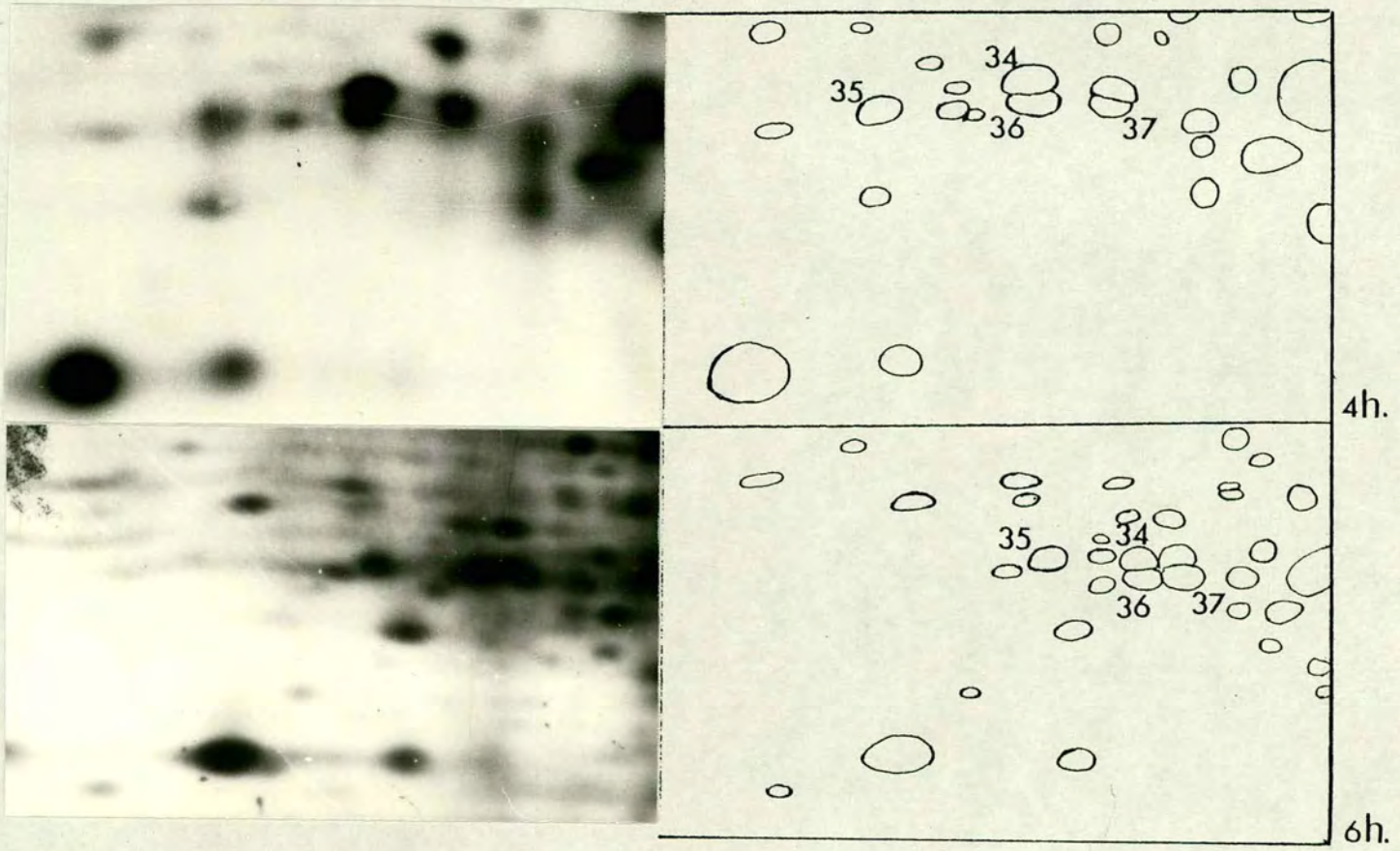


Figure 2.8B

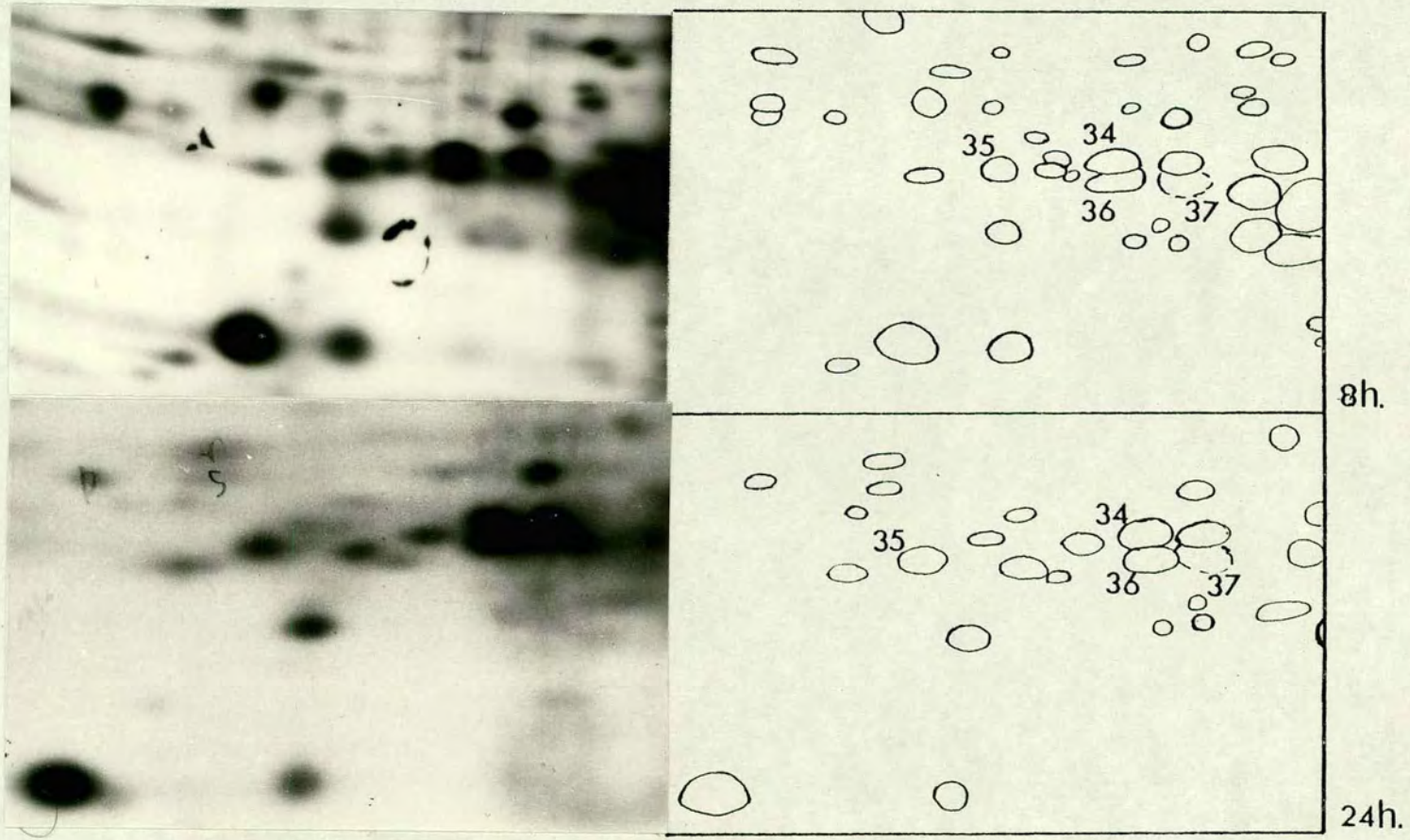


Figure 2.8 C

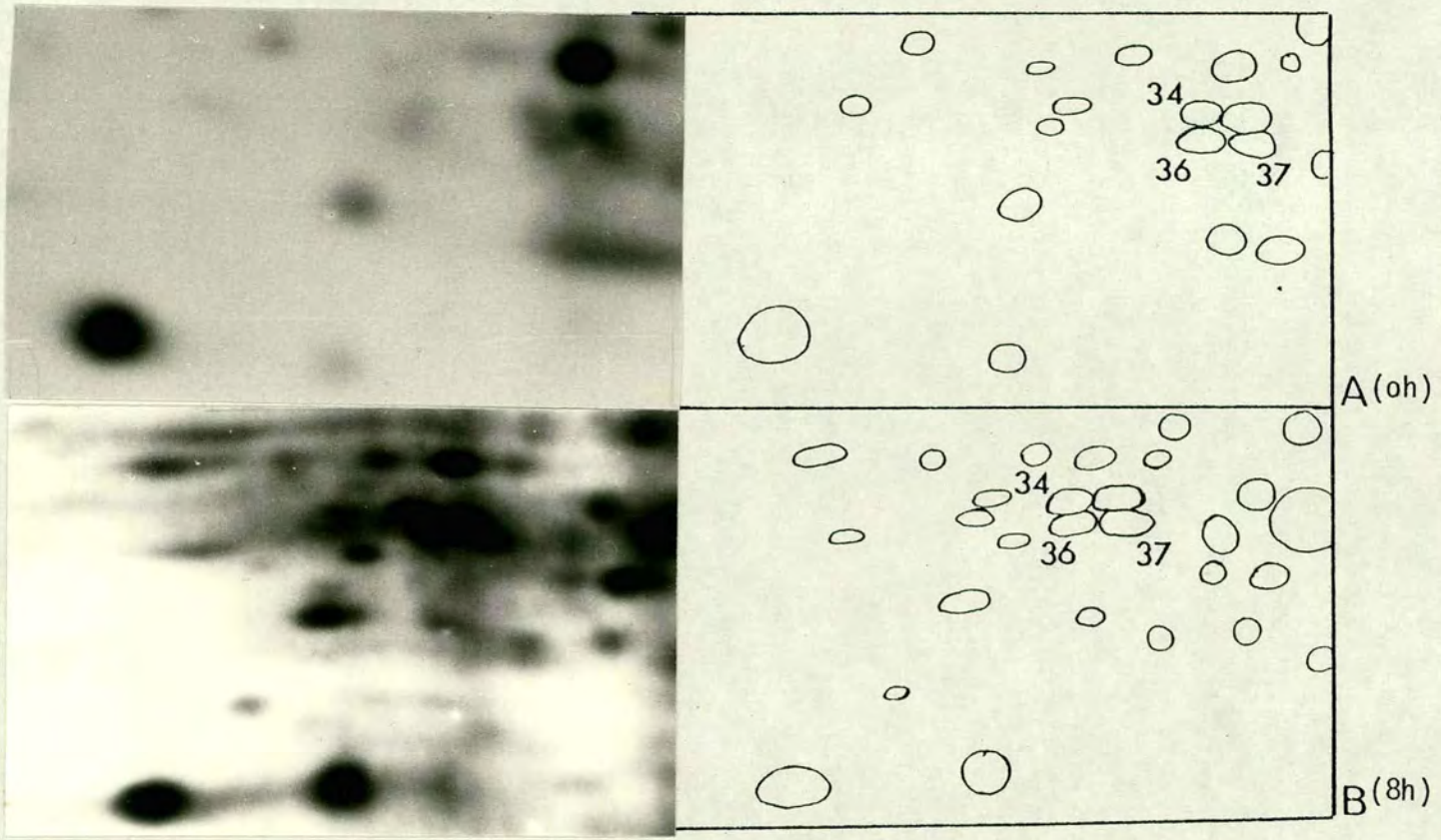


Figure 2.9: Region including polypeptides 34 to 37 from autofluorograms obtained from 0h (A) and 8h (B) extracts of the nonsporulating a/a diploids. 35 not present, 34 and 36 increased as in the a/α sporulating diploid while 37 did not increase significantly.

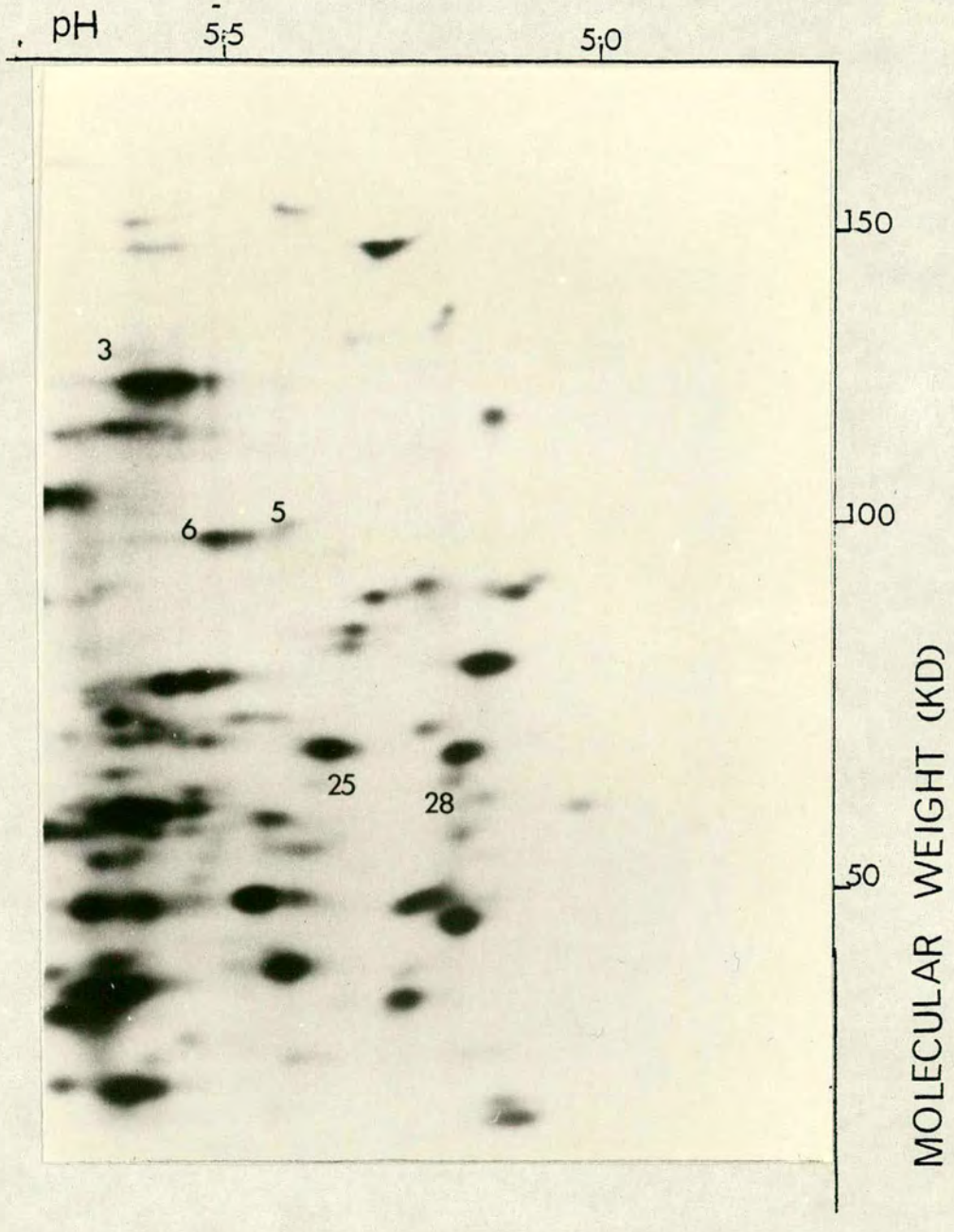


Figure 2.10A : Section of autofluorogram of [^{35}S] - labelled proteins from a/α vegetative culture. Numbered spots correspond to polypeptides that showed changes during sporulation. Sporulation-specific polypeptides were not present.

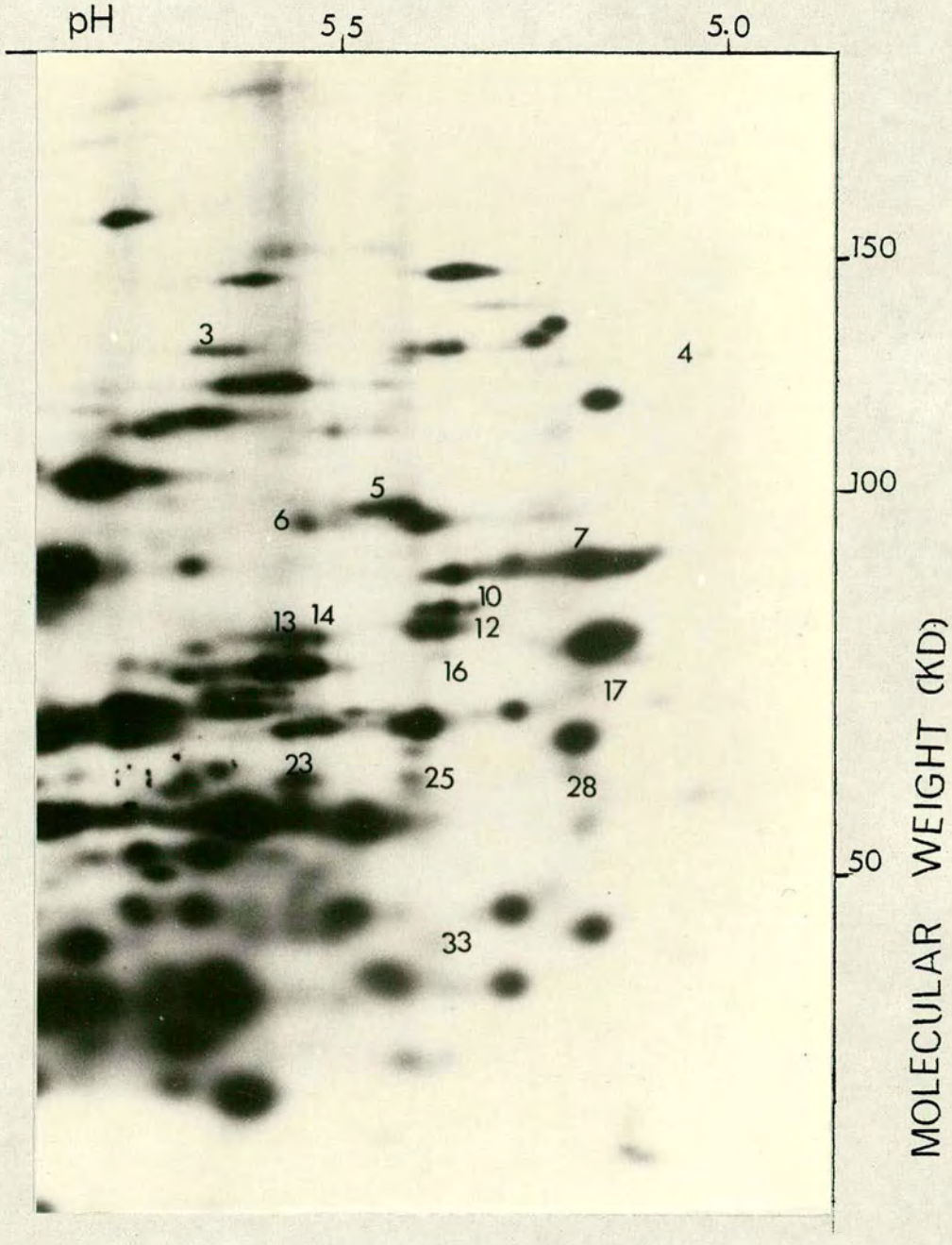


Figure 2.10B : Equivalent section as Figure 2.10A but [^{35}S] - labelled proteins from a/α 4h sporulating culture. Numbered spots correspond to polypeptides that showed changes (Refer to Table 2.1).

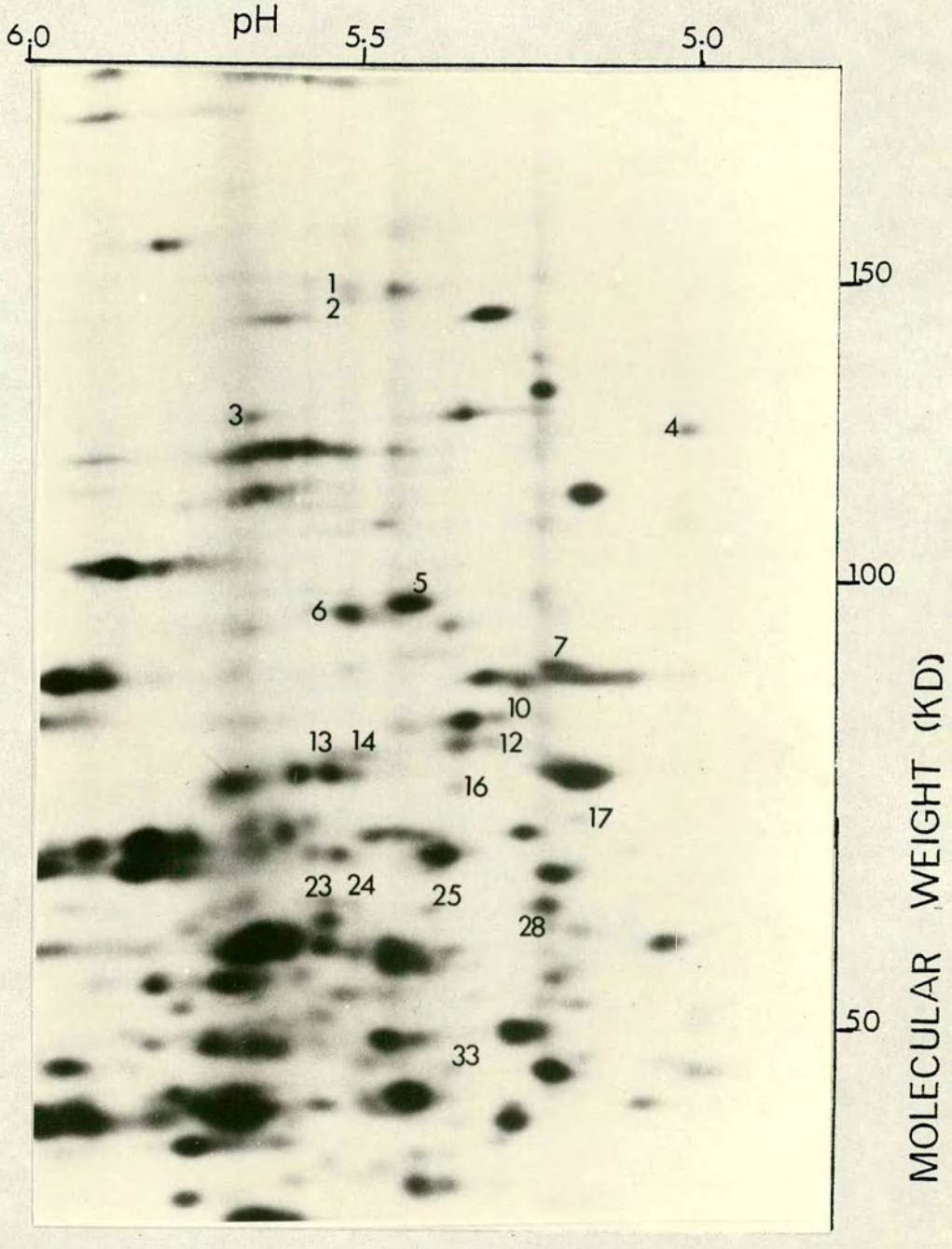


Figure 2.10C : Equivalent section as Fig. 2.10A and Fig. 2.10B but [^{35}S] - labelled proteins from a/α 24h sporulating culture. Numbered spots correspond to polypeptides that showed changes (Refer to Table 2.1). The gels for Figures 2.10A and 2.10C were exposed to X-Ray films for 7d. For Figure 2.10B, the exposure time was 8d.

4h. There was no apparent difference in timing between the appearance of new polypeptides and those that underwent an increase in concentration.

Most of the new polypeptides that appeared in both sporulating and non-sporulating strains did so within 2h of transfer to sporulation medium, but the majority of changes involving an increase in polypeptide already present in vegetative cells took place later, between 4h and 8h, and most of the other types of alteration (6, 8, 9, 15, 25, 42) occurred even later.

Discussion

Separation of protein extracts from sporulating cultures of Saccharomyces cerevisiae on one-dimensional gel electrophoresis failed to show any "sporulation-specific" proteins. As has been suggested, (Hopper et al., 1974) this is probably due to there being few true meiosis and sporulation-specific proteins and their low concentration compared to other newly synthesised proteins.

However, the results from the two-dimensional gel electrophoresis confirmed and extended those of Wright & Dawes (1979) and have shown that changes in proteins that are specific to the process of sporulation in yeast can be detected using continuous presporulation labelling techniques, and that there appears to be definite control over the timing of these changes.

Many of the changes, both sporulation-specific and those common to both sporulating and non-sporulating a/a diploids (probably resulting from the change in media) were noticeable within the first six hours after transfer to the sporulation medium. Mature asci did not appear under the same conditions until twelve to fourteen hours after resuspension, and by comparison with the time course of sporulation events obtained in the experiment and by others, these early sporulation-specific changes were taking place before cells became committed to meiosis and corresponding to the periods when DNA synthesis and recombination were occurring (Figure 2.3 and Tingle et al., 1973). They also coincide with the times at which rates of synthesis of RNA and protein are maximal (Magee, 1974). Despite this preponderance of early changes there are nonetheless a number of later

ones that are important since they define the sequential nature of the changes and provide useful markers of the later stages of sporulation. At least one of these changes may be concerned with a spore-surface protein (Briley et al., 1970) which may be the antigenic determinant reacting with antibody prepared against whole spores (Snider & Miller, 1966).

Recently, biochemical studies of yeast sporulation have attempted to find specific proteins that are synthesised uniquely during sporulation to provide the means of analysing the control of gene expression during a simple process of eukaryotic cell development. The results of several studies using pulse-labelling with L-(^{35}S)-methionine and subsequent PAGE separation of the labelled proteins were remarkable in that they appeared to show that while there are changes in the patterns of proteins synthesised in cells in sporulating conditions, the same changes occurred in sporulating a/α diploids, and non-sporulating a/a, α/α diploids and a and α haploid cells (Hopper et al., 1975; Petersen et al., 1979; Trew et al., 1979).

It is premature to suggest from these results that there are no detectable changes in gene expression that are uniquely concerned with the process of sporulation in yeast, and that all of the changes reported here are due to protein modification rather than to de novo synthesis. Pulse labelling has been done using (^{35}S)-methionine, and therefore would not have detected changes in proteins containing few methionine residues. Moreover, sporulating cells raise permeability barriers (Mills, 1972) and non-sporulating cells present in sporulating yeast population can take up some amino acids preferentially as indicated in Section 1. Recently, however, a repetition of the

pulse-labelling and two dimensional electrophoresis approach has detected one a/α specific sporulation change and in this study the uptake of the labelled methionine by sporulating cells was apparently confirmed (Kraig & Haber, 1980). There are nonetheless two changes that are specific to a/α cells during sporulation that may involve de novo enzyme synthesis; one is concerned with the α-glucosidase enzyme degrading glycogen (Claney et al., 1980; Colonna & Magee, 1978), the other with β-glucanase (del-Rey et al., 1979). Whether these, and any other sporulation-specific enzymes, are synthesised in amounts detectable using the present gel separation techniques remained to be determined.

It does seem likely that as far as the more abundant proteins of the cell are concerned, that modification may play an important part during sporulation. Modifications could include selective proteolysis methylation, adenylation and phosphorylation, among others. Phosphorylation has been examined in our laboratory and it has been shown that a few of the sporulation-specific proteins are phosphorylated or dephosphorylated during sporulation (Wright et al., 1981). The findings do not preclude the possibility that de novo synthesised proteins are involved in these modifications, but they do indicate that phosphorylation may have a role to play in sporulation.

SECTION III

ISOLATION AND CHARACTERISATION OF SPORULATION MUTANTS

In the previous section, two-dimensional gel electrophoresis of protein extracts from sporulating cultures of Saccharomyces cerivisiae showed that specific proteins are involved in sporulation and there seemed to be definite control over the timing of their appearances. Similar analysis of sporulation mutants should indicate how relevant these changes are and extend the understanding of the control and organisation of gene expression during sporulation as has already been shown in sporulating prokaryotes, such as Bacillus subtilis (Piggot & Coote, 1976). This information could be used to correlate the biochemical and morphological events of sporulation.

Mutants of S.cerevisiae that are asporogenous have been obtained by Esposito et al., (1972). However these have not been made generally available, and they are also temperature-sensitive in their sporulation defects which may be a disadvantage for the above type of analysis. Therefore more are needed, especially totally asporogenous mutants.

There is a problem in isolating mutants carrying recessive mutations affecting meiosis and sporulation, since sporulation is only expressed in diploids. To avoid this problem, Esposito & Esposito (1969) used ultraviolet (UV) or X-ray mutagenesis of haploid ascospores of homothallic strains; outgrowth of the treated ascospores led to the formation of diploids expressing any mutation induced by the mutagens. The diploids were then screened for asporogeny. Unfortunately UV and X-ray mutagenesis is relatively inefficient and since it is

difficult to screen for asporogenous mutants in S. cerevisiae, some technique for enhancing the frequency of mutagenesis by using other mutagenic agents is desirable. A modification of the Esposito's technique has been used in this laboratory to isolate spd and spo mutants. This involves ethyl methane-sulphonate (EMS) treatment of a homothallic diploid population during vegetative growth (in presporulation medium), further incubation under vegetative growth conditions to allow recovery and expression, and then sporulation of the EMS-treated population. In this way recessive mutations can be recognised in the diploid progeny arising from outgrowth of the homothallic haploid ascospores.

Several sporulation mutants were isolated using various mutagens. One, XN129 homozygous for spo 52 mutation isolated by the method of Esposito & Esposito (1969) was selected for further analysis. XN129 was completely asporogenous and was not temperature sensitive. Together with XN129, another mutant 69.10C homozygous for the spo 50 mutation has been characterised more extensively by two-dimensional gel electrophoresis. The spo 50 mutation was chosen because it causes suppression of derepressed sporulation in spd 1 strains (Dawes, 1975). It was derived from a diploid strain homozygous for the spd 1 mutation. The diploid parent strain was treated with EMS in liquid medium, then plated on YEPG plates. The spd 1 mutation is characterised by poor growth and rapid and abundant sporulation in medium in which glycerol is the carbon source. The presence of the spo 50 mutation suppressed this phenotype. Sporulation of the treated strain gave ascospores carrying the two mutations (spo 50, spd 1). Germination of these spores resulted in a/α diploids with both

mutations in homozygous state. Induced gene conversion at the mating type locus allowed mating with wild type diploids. Sporulation of the resulting tetraploid gave ascospores from which a/α diploids bearing homozygous spo 50 could be selected. By virtue of its isolation in analogy with that used to obtain spo 0 mutants in B. subtilis (Guespin-Michel, 1971) 69.10C is thought to be a very early blocked mutant (Kinnaird, 1979) possibly defective in the initiation of sporulation.

In this chapter the preliminary characterisation of these mutants is described, especially for the strain XN129 which was found to be sensitive to the UV radiation as well as being asporogenous.

Growth and sporulation responses of strains 69.10C and XN129

The capacity of the two strains to grow on fermentable and non fermentable carbon sources was examined. This indicates the proper functioning of the cells especially their ability to undergo aerobic respiration, one of the main requirements for sporulation.

Figures 3.1 and 3.2 illustrate the growth of 69.10C and XN129 on all media tested. Their growth was comparable to that of the wild type (Figure 2.1) indicating that neither mutation affecting sporulation affected the strains competence to grow vegetatively.

Microscopic examination of colonies from solid sporulation medium or liquid cultures of cells exposed to sporulation conditions did not show any sign of mature asci. XN129 remained as single cells in the slightly oval shape characteristic of cells in the G1 phase, whereas 69.10C showed abnormal growth, forming elongated cells which tended to remain attached to each other and this resulted in clumping. This phenotype was also exhibited towards the end of exponential growth, especially in fermentable carbon sources.

Survival of the populations of both strains was also slightly reduced in sporulation medium. Samples taken at hourly intervals showed that by 30h after resuspension into sporulation medium about 65% of XN129 and 60% of 69.10C remained viable compared to the insignificant decrease in the wild type (Figure 3.3).

Genetic analysis of 69.10C and XN129

The failure of the strains to sporulate made genetic analysis

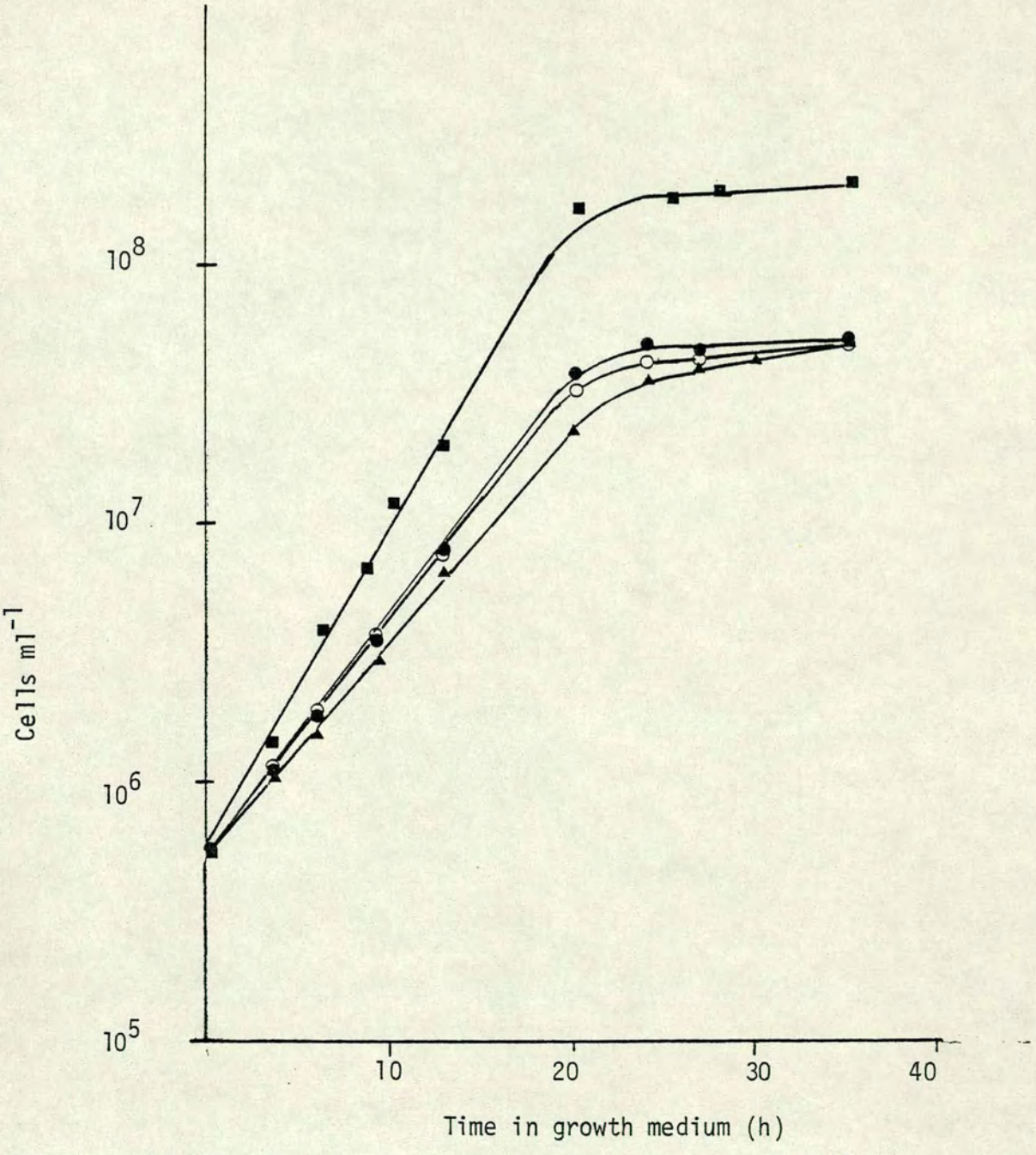


Figure 3.1 : Growth of the asporogenous diploid strain 69.10C in various media. Cells were grown in liquid media at 30°C with shaking, samples were taken at times indicated. (■) YEPD, (●) YEPA (○) YEPG, (▲) low-sulphate medium.

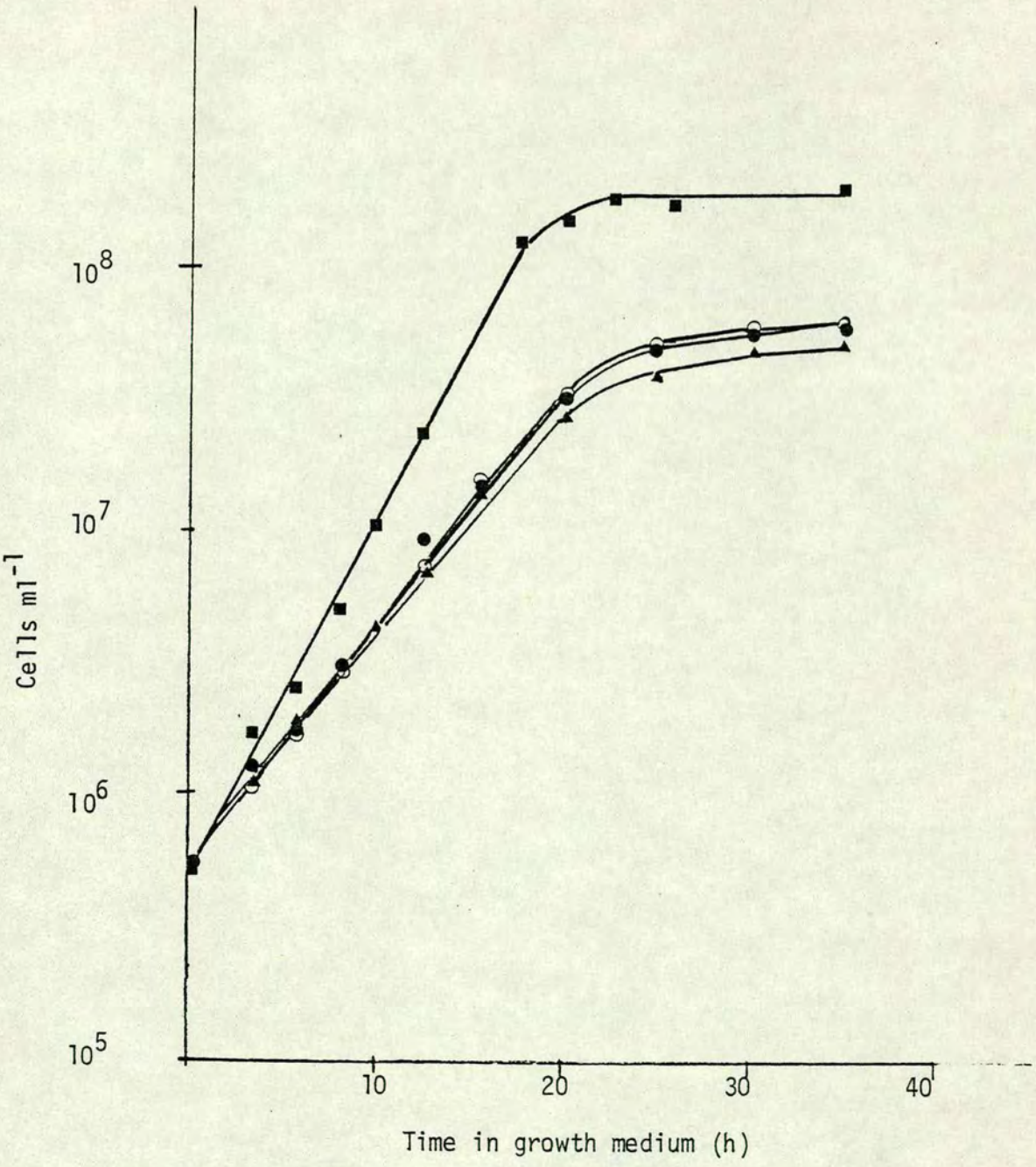


Figure 3.2 : Growth of the asporogenous diploid strain XN129 in various media. Cells were grown in liquid media at 30°C, with shaking, samples were taken at times indicated, (■) YEPD, (●) YEPA, (○) YEPG, (▲) growth of XN011 in low sulphate medium (XN129 was not able to grow in low sulphate medium [see Section IV] a derivative, XN011 was used in all experiments involving growth in low-sulphate medium.

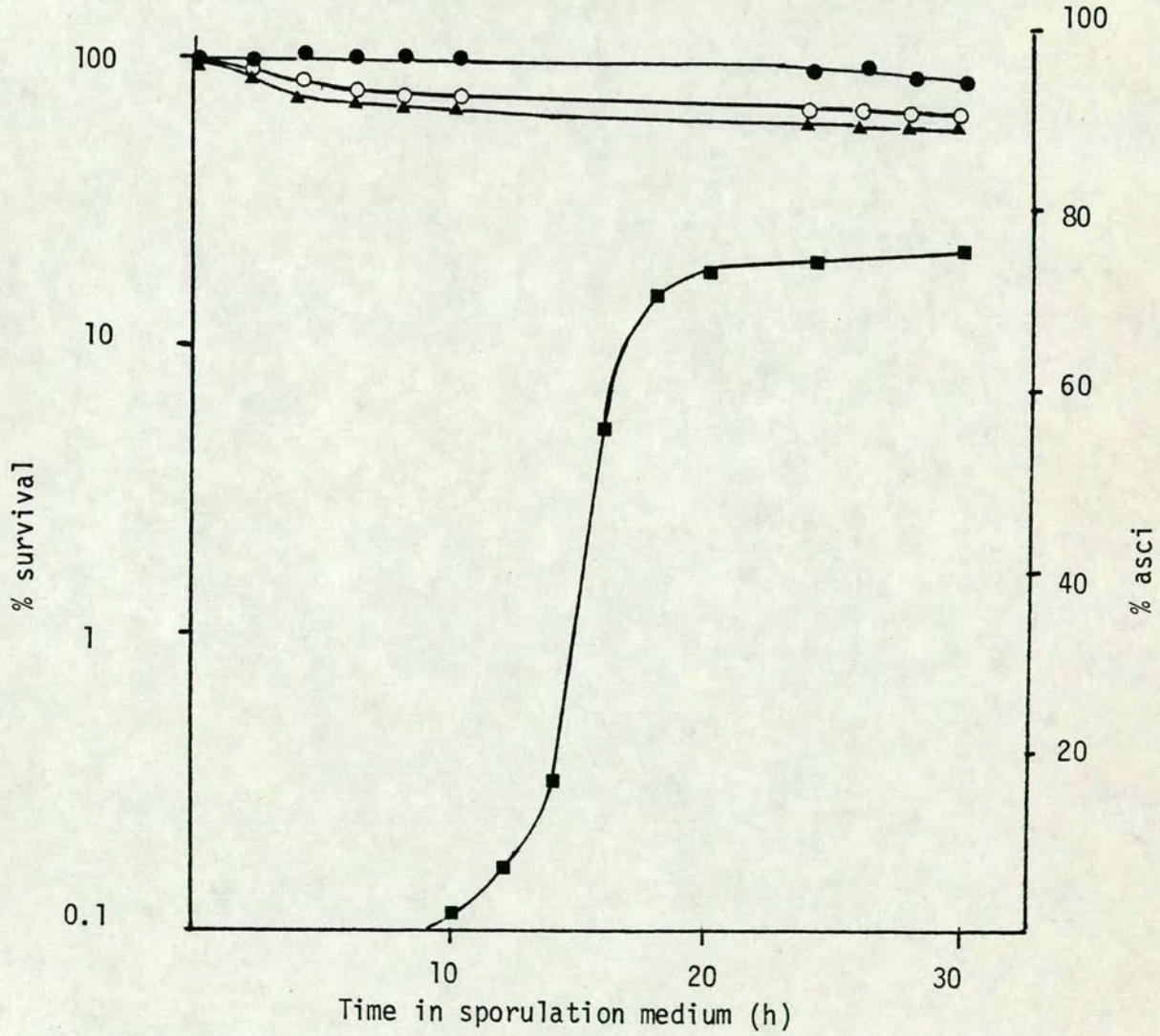


Figure 3.3 : Survival of wild type and asporogenous mutants in sporulation medium. Cells were pregrown in YEPA to a turbidity of 1 (600nm). They were centrifuged and resuspended into sporulation medium, incubated at 30°C with shaking. Samples were taken at times indicated. Percentage asci was estimated by direct counting of asci in the phase contrast microscope in several non-overlapping fields . (●) 135.11B (wild type), (○) XN129, (▲) 69.10C, (■) sporulation ability of 135.11B.

impossible by the usual meiotic analytical technique. This problem was overcome by employing tetraploid analysis. Both strains were UV-irradiated for about 15% to induce gene conversion at the mating-type locus. The process was tedious not only because of the extensive screening, but also since 69.10C has a high reversion rate and XN129 was found to be sensitive to UV-irradiation. a/a and α/α derivatives of 69.10C and XN129 were crossed to a/α sporulation-competent strains bearing complementary markers which had been similarly converted at the mating-type locus. The resultant tetraploids were selected on minimal medium and exposed to sporulation conditions. All the tetraploids formed in these crosses sporulated indicating that the mutations in 69.10C and XN129 were recessive. Spores from these crosses were dissected and typical results are shown in Table 3.2 for (69.10C x 5E-D) and Table 3.1 for (XN129 x XN02).

A tetraploid carrying gene A in duplex state (A/A/a/a) is expected to form asci that segregate 4:0, 3:1 and 2:2 for the phenotypic characteristic determined by the dominant allele and the recessive allele respectively. The genotypes of the spores in a 4:0 ascus cell all are A/a. In a 3:1 ascus, they are A/A, 2 A/a and a/a, while in a 2:2 ascus two of the spores are A/A and two are a/a. For a gene that recombines freely with its contromere the expected relative frequencies of 4:0, 3:1 and 2:2 asci are 4:4:1. The corresponding ratios for a gene located at the centromere are 2:0:1. Intermediate ratios are obtained for genes showing partial contromere linkage (Mortimer et al., 1969).

Linkage tests with tetraploid analysis of this type are not easy, especially when dealing with recessive mutations affecting sporulation

Table 3.1 : Tetraploid meiotic analysis of the asporogenous diploid strain XN129 (a/α arg4/arg4 ura3/ura3 met14/met14 spo52/spo52) X XNO2 (a/α ARG4/arg4 LEU3/leu3 MET14/met14 trp1/trp1)
Both strains were UV irradiated to induce gene conversion at the mating type locus, crossed and sporulated. Asci were dissected and results tabulated.

	ARG	LEU	MET	TRP	URA	YEPG	Mating a	Mating α	Mat. Type	UV *	KAc. ▼	SPO: spo
1 A	-	+	-	-	-	+			a/α	+-	+	4:0
B	+	+	+	+	+	+			a/α	+-	+	
C	-	+	-	+	+	+			a/α	+-	+	
D	-	+	-	-	-	+			a/α	+-	+	
2 A	+	+	+	+	+	+			a/α	+	+	2:2
B	+	+	+	+	+	+			a/α	+	+	
C	-	+	-	+	+	+			a/α	-	-	
D	-	+	-	+	+	+			a/α	-	-	
3 A	-	-	-	-	-	+			a/α	+	+	2:2
B	+	+	+	+	+	+			α/α	-	-	
C	+	+	-	-	-	-		+	a/a	-	-	
D	-	+	-	+	+	+			a/α	+	+	
4 A	-	+	-	+	+	+	+		α/α	+	-	2:2
B	-	+	-	-	+	+			a/α	+	+	
C	+	+	+	+	+	+			a/a	-	-	
D	-	+	-	+	1	+			a/α	+-	+	
5 A	-	+	-	-	-	+			a/α	+	-	0:4
B	+	+	+	+	+	+		+	a/a	+-	-	
C	-	+	-	-	+	+			a/α	-	-	
D	+	+	-	+	-	+	+		α/α	+-	-	
6 A	+	+	+	+	+	+			a/α	+	+	1:3
B	-	+	-	-	-	+		+	a/a	-	-	
C	+	+	-	+	+	+			a/α	-	-	
D	-	+	+	+	-	+	+		α/α	+	-	
7 A	-	-	-	-	+	+			a/α	+	+	2:2
B	+	+	+	+	-	+				-	-	
C	+	+	+	+	+	+				+-	-	
D	-	+	-	-	+	+			a/α	+-	+	
8 A	+	+	+	+	+	+			a/α	+-	-	2:2
B	-	+	-	-	-	-			a/α	+-	+	
C	-	+	-	-	-	+			a/α	+-	-	
D	+	+	+	+	+	+			a/α	+-	+	
9 A	-	+	-	+	+	+			a/α	+	+	3:1
B	+	+	+	+	+	+			a/α	+-	-	
C	-	+	-	-	-	-			a/α	+-	+	
D	+	+	+	+	+	+			a/α	+	+	
10 A	-	+	+	+	+	+			a/α	+-	+	2:2
B	-	-	-	-	+	+				+-	-	
C	-	+	-	+	+	+			a/α	+-	+	
D	+	+	+	+	+	+				+-	-	

		ARG	LEU	MET	TRP	URA	YEPG	Mating		Mat. Type	UV	KAc.	SPO:spo
								a	α				
11	A	-	+	+	+	-	+			a/α	+-	+	2:2
	B	-	+	-	+	+	+			a/a	+-	-	
	C	+	+	+	+	+	+			a/α	+-	+	
	D	+	+	-	+	-	+	+		α/α	+-	-	
12	A	+	+	+	+	+	+		+	a/a	+-	-	2:2
	B	-	+	-	+	-	+			a/α	+-	+	
	C	-	+	-	+	+	+			a/α	+-	+	
	D	+	+	+	+	+	+	+		α/α	+-	-	

Overall ratio 1 SPO: 1 spo

* + indicates lack of UV-sensitivity
▼ + indicates ability to sporulate

Table 3.2 : Tetraploid meiotic analysis of the asporogenous diploid strain 69.10C (a/α arg4/arg4 ura3/ura3 spo50/spo50) x 5E-D (α/α ARG4/arg4 his5/his5 LEU2/leu2 lys2/lys2 URA3/ura3 spd1/spd1 TRP1/trp1) 69.10C was UV irradiated to induce gene conversion at the mating type locus, crossed to 5E-D, allowed to sporulate, asci dissected and results tabulated.

	ARG	LEU	HIS	LYS	YEPG	Mating a	Mating α	Mat. Type	KAc. ▼	SPO:spo
1 A	+	+	+	+	+			α/α	-	0:4
B	-	+	+	+	+		+	a/a	-	
C	+	+	+	+	+		+	a/a	-	
D	-	+	+	+	+			α/α	-	
2 A	-	+	+	+	+			a/α	+	4:0
B	-	+	+	+	+			a/α	+	
C	-	+	-	-	+			a/α	+	
D	+	+	+	+	+			a/α	+	
3 A	-	+	-	-	+			a/α	+	4:0
B	-	-	+	+	+			a/α	+	
C	+	-	+	+	+			a/α	+	
D	-	+	+	+	+			a/α	+	
4 A	-	+	-	+	+			a/α	-	1:3
B	-	-	+	+	+		+	a/a	-	
C	+	+	-	+	+	+		α/α	-	
D	+	+	+	+	+			a/α	+	
5 A	-	+	-	-	+			a/α	+	2:2
B	-	+	+	-	+	+		α/α	-	
C	+	+	+	+	+			a/α	+	
D	+	+	+	+	+			a/a	-	
6 A	+	+	+	+	+	+		α/α	-	0:4
B	-	+	+	+	+		+	a/a	-	
C	+	+	+	+	+		+	a/a	-	
D	-	+	+	+	+			α/α	-	
7 A	+	+	+	+	+		+	a/a	-	2:2
B	-	+	+	+	+			a/α	+	
C	+	+	-	+	+			a/α	+	
D	-	+	+	-	+	+		α/α	-	
8 A	-	-	+	-	+			a/α	-	3:1
B	-	+	+	+	+			a/α	+	
C	-	+	+	+	+			a/α	+	
D	+	+	+	+	+			a/α	+	
9 A	+	+	+	-	+			a/α	+	1:3
B	-	+	+	-	+					
C	-	+	+	+	+					
D	-	+	+	+	+		+	a/a		
10 A	-	+	+	+	+			a/α	+	4:0
B	-	+	+	+	+			a/α	+	
C	+	+	+	+	+			a/α	+	
D	+	+	-	+	+			a/α	+	

		ARG	LEU	HIS	LYS	YEPG	Mating		Mat. Type	KAc.	SPO:spo
							a	α			
11	A	+	+	+	+	+				-	2:2
	B	+	+	+	+	+			a/α	+	
	C	-	+	+	+	+			a/α	+	
	D	-	+	+	+	+				-	
12	A	+	+	+	+	+	+		α/α		2:2
	B	-	+	+	+	+			a/α	+	
	C	-	-	-	-	+			a/α	+	
	D	+	+	+	+	+		+	a/a		

Overall ratio 1 SPO : 1 spo

▼ + indicates ability to sporulate

(since they are only expressed in a/α homozygous diploids) and with mutations affecting radiation resistance for which heterozygous diploids may show an intermediate level of resistance.

The crosses involving 69.10C and XN129 have a number of known factors that contributed to the asporogeny of the ascospores. From Tables 3.1 and 3.2, the final ratios of ascospores showing sporogenous and asporogenous phenotypes were 1:1. There was no evidence to indicate how close the asporogenous genes were to their respective centromeres. Since they are recessive to the wild type (and assuming they are located a fair distance from the centromere) the ratio of sporogenous to asporogenous progeny due to the spo mutation was 5 Spo^+ : 1 Spo^-. The ability to sporulate is, however, also determined by the mating type locus, only a/α ascospores sporulate, the frequency of sporulating ascospores due to this locus is 2 in 3 (2:1 ratio) Taking into account both genes the expected frequency is 10 Spo^+ : 9 Spo^- (a/α and SPO have to be present together in order for the spore progeny to sporulate, the presence of either spo or a/a, α/α will block sporulation). Thus the observed ratios agreed with the expected ratios for single gene mutations causing the spo phenotype in the mutant strains.

The radiation-sensitive, asporogenous strain XN129

The analysis of strain XN129 was complicated by the radiation sensitivity and one of the first problems that needed to be resolved was whether or not the recessive mutation causing radiation sensitivity was linked to the recessive mutation to asporogeny. From the data in Table 3.1 it is clear that this was indeed the case, since after meiotic analysis of the cross of XN129 to a radiation resistant, sporulating

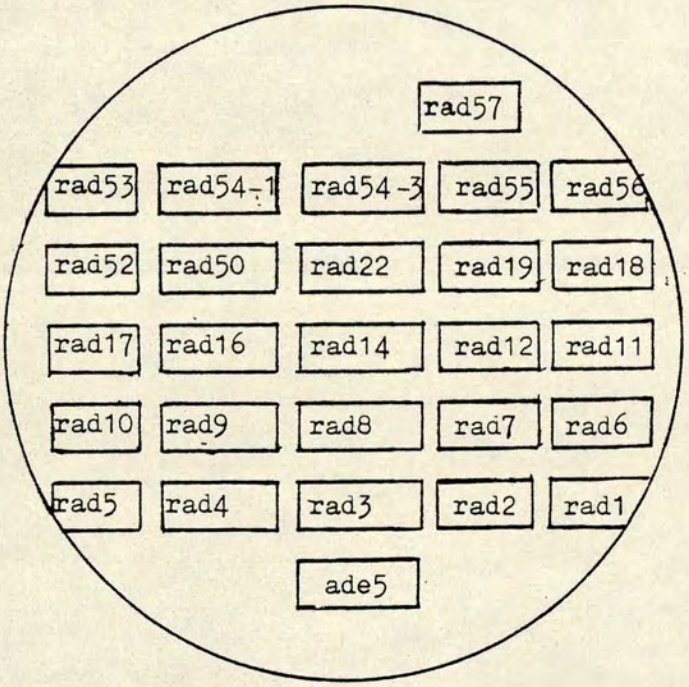
diploid, all spore progeny in which the genes could be assigned unambiguously retained the parental combinations of radiation resistance and ability to sporulate. Therefore it is very likely indeed that the two phenotypes are the result of a single mutation. It should be noted that the mutation(s) was induced by UV and closely linked double mutations were not expected. Moreover the asporogeny was due to a single mutation of a nuclear gene and no evidence of centromere linkage was observed.

Identification of the UV radiation sensitive mutation in XN129

It was an interesting observation that the sporulation ability of ascospores from tetraploid crosses involving XN129 were influenced by the segregation of the UV-sensitive gene which appeared to be partially dominant (Table 3.1). All those spores that were insensitive or semi-sensitive to UV radiation were able to sporulate. Sporulation however varied, ranging from 70% to as low as 5%. Spores that were very sensitive to UV radiation failed to sporulate.

In order to investigate the role of the UV sensitive gene during sporulation, attempts were made to identify the mutation in strain XN129 by screening in a complementation test against all available mutations to radiation sensitivity. $\underline{a}/\underline{a}$ and $\underline{\alpha}/\underline{\alpha}$ revertants of XN129 were crossed to the 54 $\underline{a}$ and $\underline{\alpha}$ radiation-sensitive strains obtained from the Yeast Genetic Stock Centre, University of California, Berkeley. The triploids formed were replicated onto YEPD plates and exposed to UV radiation. After 5d growth at $30^{o}C$, the crosses were scored for growth. Figure 3.4 illustrates the results seen on YEPD plates. The mutation XN129 complemented all mutations

Figure 3.4 : Complementation test of the gene for radiation-sensitivity in strain XN129 with other radiation-sensitive genes. 27 radiation-sensitive strains (a, haploid) from the Berkeley Stock Centre were streaked on YEPD plates, incubated overnight then replica-plated onto YEPD plates which had been spread with a lawn of an α/α derivative of XN129. They were allowed to mate overnight, replica-plated onto YEPD plates which were then exposed to UV radiation (1.05 J s^{-1} m^{-2}) for (A), 0s, (B), 15s and (C) 30s. Photographs were taken after 2d growth in the dark at 30°C. The pattern of the radiation-sensitive (rad) strains on the master plate is illustrated below. Only the strain bearing the rad 3-2 gene failed to complement the radiation-sensitivity of the XN129 strain. Note this experiment involves testing in the triploid state i.e. for the cross; a rad 1 (etc.) x $\frac{\alpha \text{ spo52 (rad?)}}{\alpha \text{ spo52 (rad?)}}$



A

B

C

conferring radiation sensitivity that were tested except rad 3-2.

Responses of rad 3-2 and spo 52 bearing strains to UV radiation

Strain XN129 and diploids homozygous and heterozygous for rad 3-2 were plated on YEPD plates and exposed to UV radiation ($1.05 J\ s^{-1}\ m^{-2}$). Figure 3.5 shows their responses compared to the wild type strain. The presence of a wild type allele in diploids heterozygous for rad 3-2 did not completely eliminate the sensitivity to UV radiation. Diploids homozygous for rad 3-2 were slightly more UV-sensitivity than XN129. The strain that resulted from crosses of XN129 and rad 3-2 showed the same response as rad 3-2 diploids. Note however that these were triploids and while the results cannot be directly compared they clearly confirmed that the mutation in XN129 failed to complement that in rad 3-2.

The effect of radiation genes on sporulation ability

Table 3.3 illustrates the influence of rad 3-2 and the rad gene of XN129 on sporulation ability. Diploids homozygous for rad 3-2 were able to sporulate at levels up to 40% of the cell population. Crosses between strains XN129 and rad 3-2 did not sporulate very well, at frequencies ranging between 5-20%; therefore the presence of both mutations in heterozygous condition did not block sporulation completely.

Ability of XN129 UV radiation resistant revertants to sporulate

Several attempts were made to induce reversion of the rad 3

Table 3.3 : Sporulation ability of strains bearing rad3-2 and the presumptive rad 3 allele from strain XN129

Genotype	Sporulation ability
RAD3/RAD3	70-80%
rad3-2/rad3-2	30-40%
XN129 x wild type RAD3 /rad3-x /rad3-x	30%
rad3-2 x XN129 rad3-2 / rad3-x / rad 3-x	5-20%
XN129 rad3-x /rad3-x	0%

Strains were grown in YEPA, to a turbidity of 1 (600nm), centrifuged and immediately resuspended into sporulation medium. Sporulation ability was estimated after 24h incubation at 30°C by direct counting of asci in the phase contrast microscope (X400) in several non-overlapping fields.

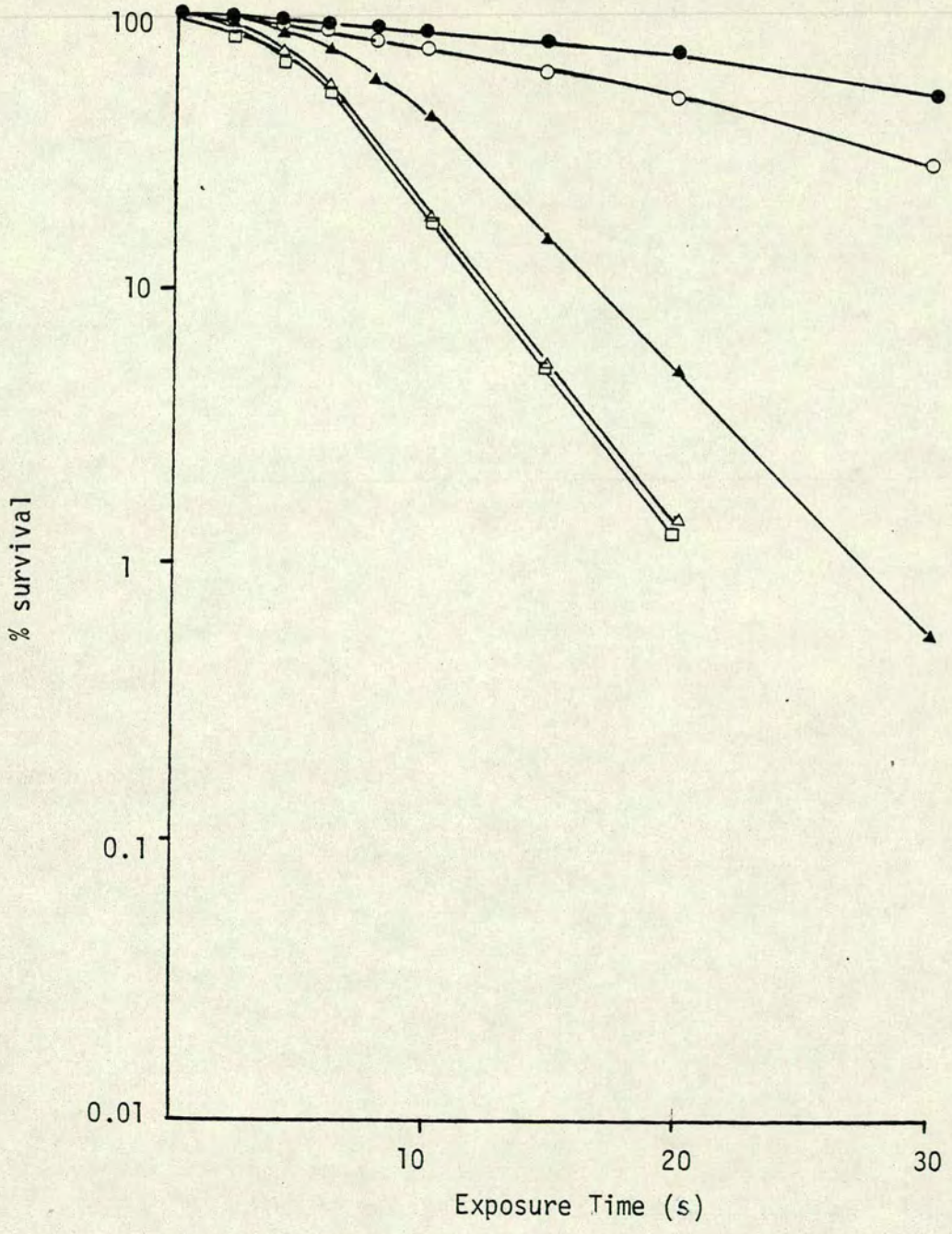


Figure 3.5 : Responses of the wild type and the UV radiation-sensitive strains to various doses of UV radiation. Strains were appropriately diluted and spread on YEPD plates, they were allowed to dry at 30°C for 2h; then exposed to UV radiation (1.05 J s^{-1} m^{-2}) for different exposure times (at least 4 plates were allocated for each time point), (●) 135.11B (wild type), (▲) XN129, (△) rad 3/rad3, (□) rad 3 x XN129, (○) RAD3/rad3.

allele in XN129 using N - methyl - N' - nitro - N - nitrosoguanidine (NTG) and UV radiation. The mutation however seemed to be fairly stable and the few revertants obtained could be divided into two groups; those partially sensitive to UV radiation and those completely insensitive to UV radiation (Figure 3·6). The former showed a very low level of sporulation or were not able to sporulate whereas the latter sporulated very well, and were comparable to the original wild type (135-11B). This result is not incompatible with the hypothesis that the mutation in XN129 causes both asporogeny and radiation sensitivity, although the nature of the partial revertants remains to be elucidated.

Meiotic DNA synthesis in 69.10C and XN 129

Premeiotic DNA synthesis is the first recognisable biochemical event in meiosis and sporulation. Measurement of DNA content in strains 69.10C and XN129 would therefore indicate whether these two mutants were able to reach this stage, in the sequence of events that lead to spore formation.

Exponentially growing cultures of both mutant strains, of an a/a non-sporulating diploid and an a/α sporulating diploid in YEPA were resuspended into sporulation medium and incubated at 30°C. Duplicate samples were taken at hourly intervals from all the cultures and their DNA content assayed. Figure 3.7 illustrates the results during the 30h period examined. The a/α sporulating diploid showed a 65% increase in DNA concentration, while the two mutants showed a comparatively insignificant level of synthesis. Cycloheximide, which inhibits initiation of DNA synthesis indirectly by suppressing protein synthesis

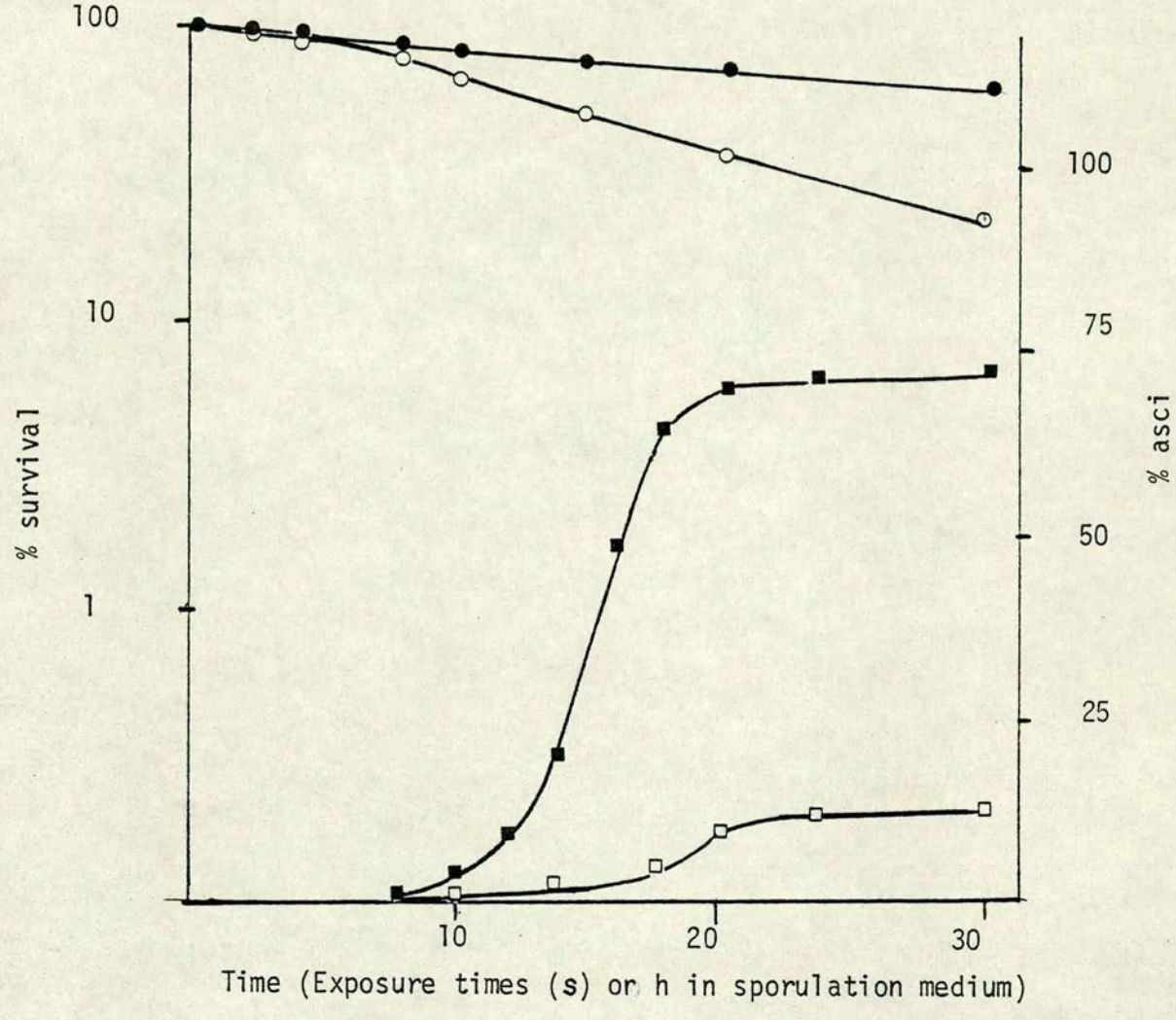


Figure 3.6 : Dose-response curves for UV irradiation of UV-sensitive revertants of XN129 and their sporulation ability. Determination of UV-sensitivity was done as for Figure 3.5 and sporulation ability was estimated as for Figure 3.3. (●) survival of group I revertants, (■) sporulation ability of group I revertants, (○) survival of group II revertants, (□) sporulation ability of group II revertants.

(Colombo et al., 1969; Pestka, 1971) was added to a culture of a/α diploid immediately after resuspension into sporulation medium. Comparing the DNA assay results from the treated culture and the two mutants indicated that there was a low level of DNA synthesis in the two mutants. Cycloheximide inhibited both mitotic and meiotic synthesis, thus the final concentration in 69.10C and XN129 included DNA resulting from mitotic synthesis before the cells reached the G1 phase. XN129 synthesised about 5% more DNA compared to 69.10C.

Commitment to recombination and segregation

Meiotic DNA synthesis is usually accompanied by high levels of genetic recombination. To test whether 69.10C and XN129 were able to progress to this stage and to the subsequent meiotic segregation, strains that were homozygous for the spo mutation concerned and hetero-allelic for two different alleles at the ade 2 and at the his 4 loci were constructed. Cells committed to recombination can be scored by testing for loss of the histidine requirement due to recombination between the non-complementary alleles of the his 4 gene complex. The ade 2-heteroalleles complement each other, the parent diploid was white and adenine independent; when segregation of the nucleai occurred, the haploid progeny required adenine and appeared as red (ade 2-40) or pink (ade 2-119) colonies. Cells committed to completing meiosis can thus be scored directly after plating on any medium. Figure 3.8 illustrates the results for the wild type strain (60 x 61). Mature asci were first observed after 16h in sporulation medium; by 30h 70% of the population had completed sporulation. The histidine-independent colonies reached a maximum after about 8h when meiotic segregation began

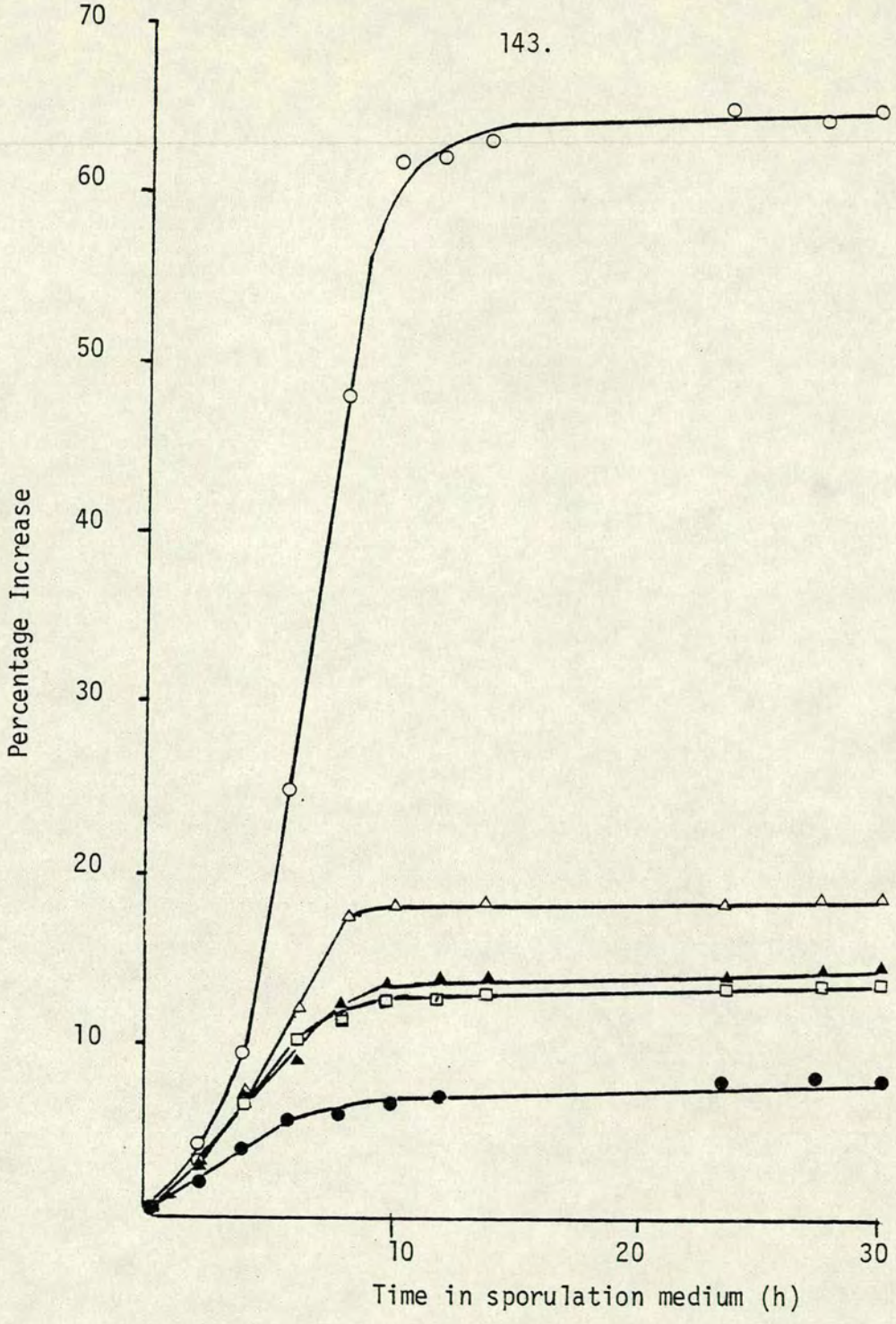


Figure 3.7 : DNA synthesis in wild type and asporogenous mutants and the effect of cycloheximide treatment. All strains were grown in YEPA harvested at a turbidity of 1 (600nm) and resuspended in sporulation medium. Duplicate samples were taken at times indicated. DNA was assayed according to the method of Stewart (1975). (○) a/α sporulating diploid (135.11B), (●) 135.11B treated with cycloheximide (100ug ml^{-1}) immediately after resuspension into sporulation medium, (△) XN129, (▲) 69.10C, (□) a/a non-sporulating diploid.

as shown by the appearance of the red and pink colonies. The derivatives of 69.10C did not show a significant increase in the number of histidine-independent colonies when the population was sampled at 2 hourly intervals during exposure to sporulation conditions, neither did it show segregation of the ade 2 heteroallic marker. Figure 3.9 showed results for the derivative of XN129, the histidine-independent colonies rose in number at about the same time as in the wild type, however they did not reach the maximum percentage obtained in the wild type. A few red and pink colonies were also observed, the number however remained low throughout the period examined.

Examination of the nuclear configuration of 69.10C and XN129 during exposure to sporulation conditions

After 4 and 8h exposure to sporulation conditions in liquid medium, samples from strains 69.10C, XN129 and 135.11B were fixed and treated with 33258 Hoechst to stain DNA containing structure (Lemke et al., 1978). Vegetative and G1 phase cells (grown in YEPD until stationary phase) were treated similarly. Microscopic examination of stained cells did not show any difference in the nuclear configuration of the two mutants at any time during exposure to sporulation conditions compared to the nuclei of G1-phase cells. Staining the nuclei with Giemsa stain gave similar results. The nuclei of cells from both strains exposed to sporulation conditions did not exhibit any changes that have been detected in the nuclei of cells undergoing sporulation.

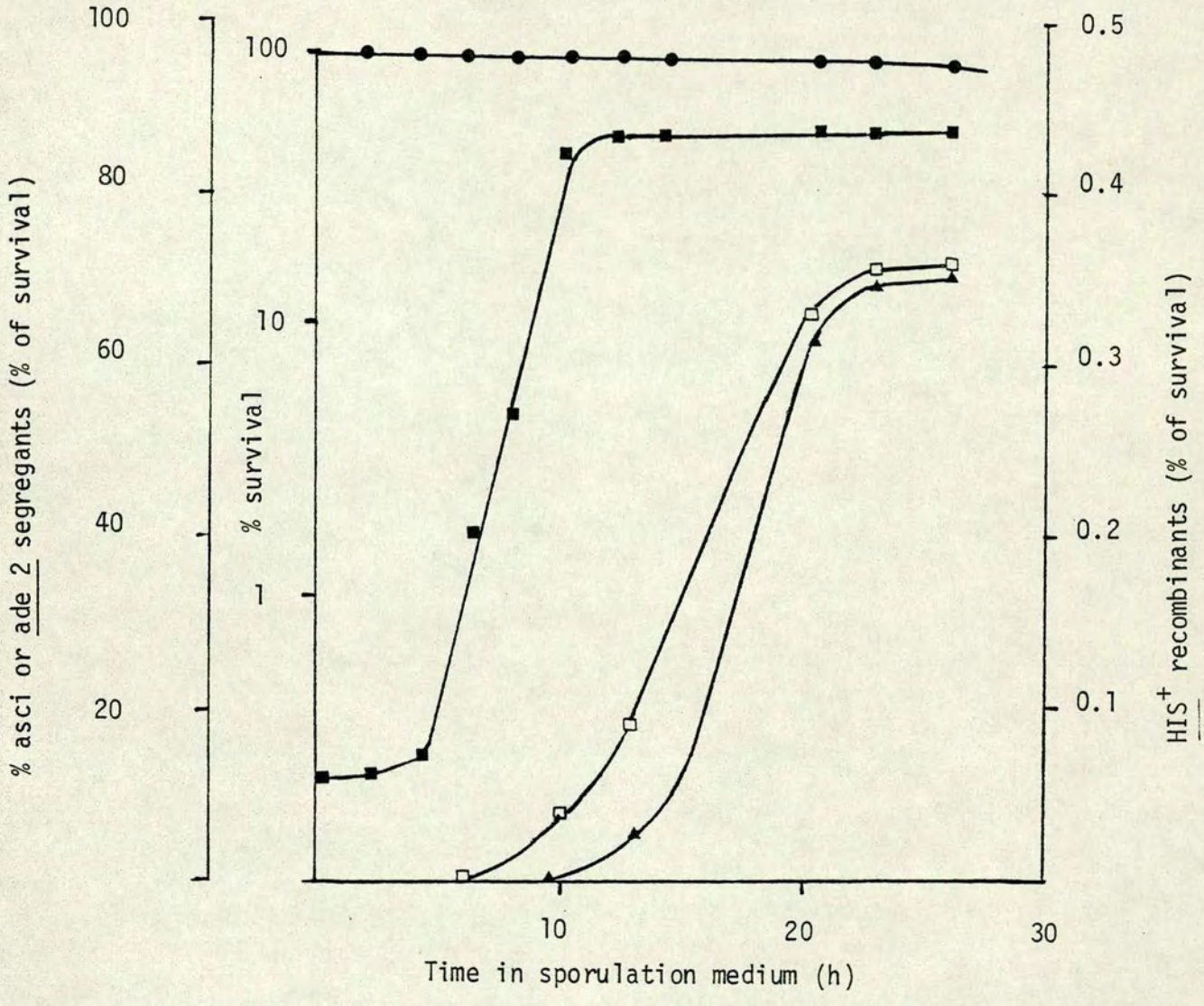


Figure 3.8 : Sequence of events during sporulation of wild type 60 x 61. (■) meiotic recombination as measured by the incidence of HIS^+ recombinants, (□) meiotic segregation as measured by ade 2 segregants (▲) % asci, (●) % survival.

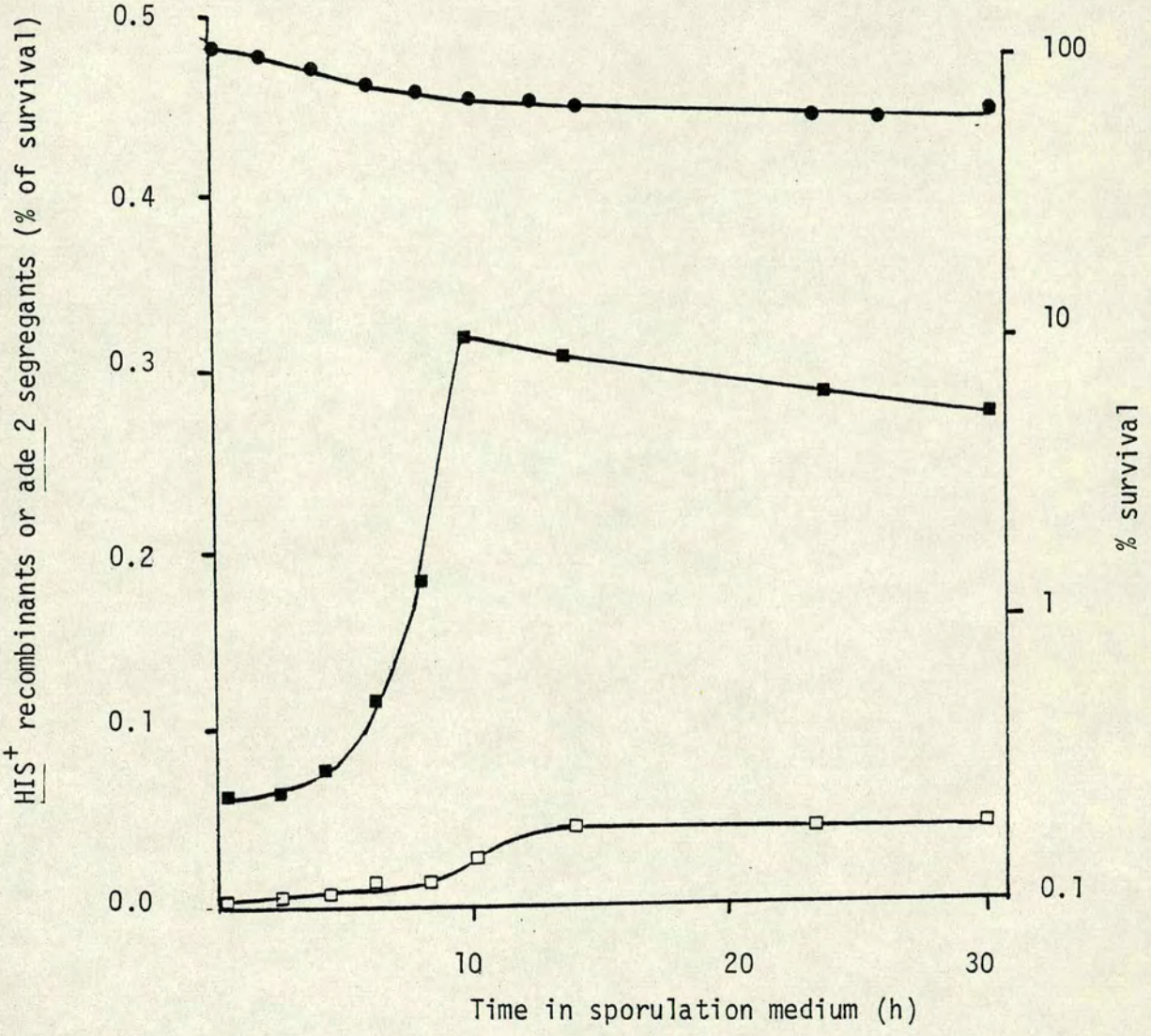


Figure 3.9 : Meiotic recombination and segregation during exposure of a diploid bearing homozygous spo 52 mutation to sporulation conditions. (■) meiotic recombination as measured by incidence of HIS$^+$ recombinants, (□) meiotic segregation as measured by ade 2 segregants. (●) % survival.

Electron microscopic examination of XN129 and 135.11B

Electron microscopy of a/α sporulating cells from various stages of sporulation has revealed characteristic changes in the nuclear structures (Moens, 1971; Moens & Rapport, 1971a). Despite its ability to initiate DNA synthesis and undergo meiotic recombination, mutant XN129 did not show any changes in nuclear configuration when stained with the nuclear stains 33258 Hoechst and Giemsa. Electron microscopic examination of thin sections of cells at various stages of sporulation of 135.11B and XN129 was therefore undertaken. While the wild type (135.11B) showed signs of spore formation and mature asci, nuclei of XN129 resuspended in sporulation medium did not show any significant difference compared to nuclei of cells at G1 phase of XN129 or 135.11B.

Discussion

The genetic data indicated that the mutations in strains 69.10C and XN129 were recessive, due to a single nuclear gene mutation and that they were specifically affecting sporulation. Vegetative growth was not affected as the strains were able to grow normally in all media tested. Apart from not being able to sporulate, XN129 was also sensitive to UV radiation; 69.10C was not.

Several rad genes have been shown to affect meiosis and sporulation (Game & Mortimer, 1974; Prakash et al., 1980). From the data presented above it is very likely that the UV radiation sensitivity mutation in XN129 is an allele of the rad 3 locus. It is clearly not the same as the rad 3-2, and some degree of complementation of the two different alleles was observed with respect to sporulation and (to a small extent) with radiation sensitivity.

Meiotic analysis, insofar as it was feasible using tetraploids, showed that the asporogenous and radiation-sensitive phenotypes of XN129 were the result of the same mutation (or very closely linked mutations). The fact that reversion to full radiation resistance was accompanied by conversion of the ability to sporulate confirms this view. If the newly isolated mutation is an allele of the rad 3 locus, it identifies another function that is involved in both radiation repair and in sporulation. Other workers have previously shown that the rad 6, 9, 50, 51, 52, 53, 54, 55, 56 and 57 genes are involved in sporulation, but the rad 3 gene product involvement has not been noted to date. It is in fact interesting that the rad 3-2 mutation does not affect sporulation as markedly as the rad 3

allele in strain XN129. One explanation of the difference between these two alleles is that the RAD 3 gene encodes at least two different functions. Both are concerned with repair of UV-induced radiation damage, but only one with sporulation.

Meiotic recombination normally occurs after initiation of meiotic DNA synthesis (Esposito et al., 1975; Haber et al., 1975). Since XN129 exhibited some recombination ability, the slightly higher DNA content (compared to 69.10C and the a/a non-sporulating diploid) must be due to meiotic DNA synthesis. The sporulation defect was thus probably expressed during recombination which involves "nicking" of DNA, formation of heteroduplexes and repair synthesis (Holliday, 1964; Meselson & Radding, 1975). The rad 3-2 mutation has been shown to cause a defect in the control of a step in excision repair of UV-induced radiation damage (Cox & Game, 1974; Game & Cox, 1973). Most likely the defect in XN129 was also in the same pathway, however unlike rad 3-2, the function expressed by the rad gene in XN129 played a vital role in meiotic recombination in XN129.

The data presented also provided evidence that commitment to recombination begins before the cells are committed to meiotic disjunction and a certain amount of meiotic DNA synthesis has to occur before recombination can begin as indicated by others (Esposito et al., 1975; Esposito & Esposito, 1974b; Haber et al., 1975).

The abnormal phenotype exhibited by 69.10C on resuspension into sporulation medium and at the end of exponential growth indicated that some control mechanism at or during the transitions from mitosis to G1 to meiosis was affected. The cells were unable to enter G1 or

meiosis and growth continued but was restricted by the limited level of nutrients.

The decline of viability of both mutants incubated in sporulation medium reflected either the loss of colony-forming ability by recombinant cells which became committed to aberrant sporulation or it reflected the physiological state of the cells. For example, it has been reported that cells lost their ability to survive when arrested in stages of the cell cycle other than the G1 phase (Hartwell, 1974).

Staining experiments indicated that sporulation in both mutants was terminated before any change occurred in the configuration of their nuclei. Fine structure studies of XN129 were consistent with the above observations, despite its later block than that of strain 69.10C. There was no evidence of spindle pole body formation for meiosis I and since this mutant exhibited recombination after exposure to sporulation conditions, commitment to recombination does not need the duplication of the spindle pole body, which agreed with the observations made by Esposito & Esposito (1974b).

The two mutations, spo 50 in 69.10C and spo 52 in XN129 have therefore been characterised in some detail, and diploids homozygous for one or other will provide suitable strains for studying the effects of genetically blocking sporulation at two different (early) stages on the sequence of changes in polypeptides that has previously been detected in the wild type.

SECTION IV

SPORULATION SPECIFIC POLYPEPTIDES PRODUCED IN ASPOROGENOUS MUTANTS AND THE EFFECT OF CYCLOHEXIMIDE ON THEIR APPEARANCES

In Section II, two-dimensional gel electrophoresis analysis of [^{35}S] - labelled polypeptide extracts prepared from a/α sporulating and a/a non-sporulating diploids at various time after resuspension into sporulation medium, showed that sporulation-specific changes occurred during sporulation. These changes were sequential, each occurring at characteristic times during sporulation.

An extension of this analysis to sporulation mutants blocked at different stages of meiosis and sporulation should indicate how relevant these changes are to the sporogenesis and the polypeptide complements of the mutants could be related to the points at which the mutants are blocked in the morphological sequence. The results would also indicate whether there is any dependence of events during sporulation i.e. whether early events have to be successfully executed before later events could be initiated. Thus, labelled protein extracts from two asporogenous mutants, 69.10C which is blocked fairly early in the process (since it failed to show any recognisable events that have been shown to accompany sporulation) and XN129, which is blocked after the initiation of meiotic DNA synthesis were analysed by two-dimensional gel electrophoresis.

The polypeptide changes detected by this method may be due to modifications of pre-existing polypeptides or de novo synthesis. Treatment of cultures with cycloheximide (an inhibitor of protein synthesis on cytoplasmic ribosomes) after resuspension into sporulation

medium should give some indication for each polypeptide which of these possibilities applies. A second analysis of labelled polypeptides from the two mutants resuspended in sporulation medium with added cycloheximide was therefore undertaken as the first step to differentiate whether the changes observed were due to modifications or de novo synthesis.

Pre-sporulation growth and labelling of cellular proteins

In the study of the wild type strains, low-sulphate medium with $^{35}SO_4^{2-}$ added was used as the pre-sporulation growth medium to label the proteins. Strains 69.10C was able to grow normally in this medium, XN129 however, failed to grow. The wild type 135.11B showed a similar defect and the inability to grow in low sulphate was traced to the methionine requirement of the strains. The pathway for sulphate assimilation has been shown to be part of the methionine biosynthetic pathway (Breton & Surdin-Kerjan, 1977). Strains bearing met 14 lack adenosine 5'-phosphate kinase which makes the cell unable to assimilate sulphate from the medium and thus inhibits growth. To overcome this inhibitory effect the met 14 marker was crossed out of strain XN129. The resulted strain (XN011) grew normally in low-sulphate medium (Figure 3.2) and the extent of label uptake was similar to that of JW1 and JW2.

Pattern of protein synthesis in 69.10C and XN011

Figures 4.1 and 4.2 show the autofluorograms developed after electrophoresis of [^{35}S] - labelled protein extracts from strains 69.10C and XN011 exposed to sporulation conditions. As before, using strains JW1 and JW2, about 400 polypeptides were detected. The general pattern of the polypeptides in both mutants was the same as seen in JW1 and JW2 with respect to those polypeptides not undergoing alterations during sporulation.

Polypeptide changes in 69.10C and the timing of their appearances

In strain 69.10C only a few of the sporulation-specific

Figure 4.1 : Autofluorogram of [^{35}S] - labelled proteins separated in the first dimension by isoelectric focussing (pH3-10) and in the second according to molecular weight in a 0.1% SDS, 5-15% polyacrylamide gel slab. The proteins are from an extract of strain 69.10C prepared after 24h in sporulation medium. The gel was exposed to X-Ray film for 6d.

Figure 4.2 : Autofluorogram of [^{35}S] - labelled proteins separated in the first dimension by isoelectric focussing (pH 3-10) and in the second according to molecular weight in a 0.1% SDS, 5-15% polyacrylamide gel slab. The proteins are from an extract of strain XN011 prepared after 24h in sporulation medium. The gel was exposed to X-Ray film for 8d.

polypeptide changes were detected. Table 4.1 illustrates the changes observed and the timing of their appearances. Of the 21 sporulation-specific changes found in the wild type only five could be detected and these (16, 20, 23, 24 and 26) were due to the appearances of new polypeptides. Four vegetative polypeptides (14, 10, 13 and 14) that in the wild type showed concentration increases were detected, however they remained unchanged in strain 69.10C and therefore the "sporulation-specific" changes did not take place (Table 4.3). The specific changes that did occur were those that appeared early, within the first 4h after resuspension into sporulation medium. Therefore in strain 69.10C which is thought to be blocked very early in sporulation, possibly in initiation (see Section III of results), very few of the sporulation-specific changes were seen to take place, and those that did would normally have occurred within the first 4 hours after resuspension.

The polypeptide appearances and alterations that in the wild type were common to both a/α sporulating and a/a non-sporulating cells were mostly detected in strain 69.10C, but there were some differences when compared with the results from wild type a/α and a/a diploids. All of the "new" polypeptides except polypeptide 29, started to decrease in concentration between 6h and 8h after resuspension into sporulation medium. Polypeptides that normally would have increased in concentration within the first 4h were not affected in the mutant but those that would have begun concentration increases at 6h remained unchanged except polypeptide 36 (Figure 4.3A).

The seven remaining polypeptides, that is, those which in the wild type involved alterations other than those listed above,

Table 4.1 : Nature and Timing of significant changes in polypeptides during exposure of 69.1OC to sporulation conditions.

Nature of change	Identity of Polypeptide* and Time of First Appearance				
	2	4	6	8	16-24h
Sporulation specific appearances	(7), (17), 20	(11), 16, 23	24, (39)		(1), (2)
- NEW	26, (27), (35)	(38)			
- Concentration Increase	[4], [13], [14] (37)	[10], (45)		(28)	
Common appearances					
- NEW	29, 40, 43 44	12	33		
- Concentration Increase	5, 18, 19	3, 31, 32	[21], [22], [30] [34], 36		
Other alteration		[41]	8, (15)	9, [42]	6, [25]

(◯) polypeptide absent, [□] polypeptide present but showed no change

* Polypeptides are numbered according to the scheme given in Figure 2.7.

Figure 4.3 : Sections, including polypeptides 34 to 37 from autofluorograms of [^{35}S] - labelled polypeptides of (A) 69.10C and (B) XN011 after 8h in sporulation medium. Polypeptides 35 and 37 were not present in 69.10C. All gels for Figures 4.3, 4.4 and 4.5 were exposed to X-Ray films for 7 d.

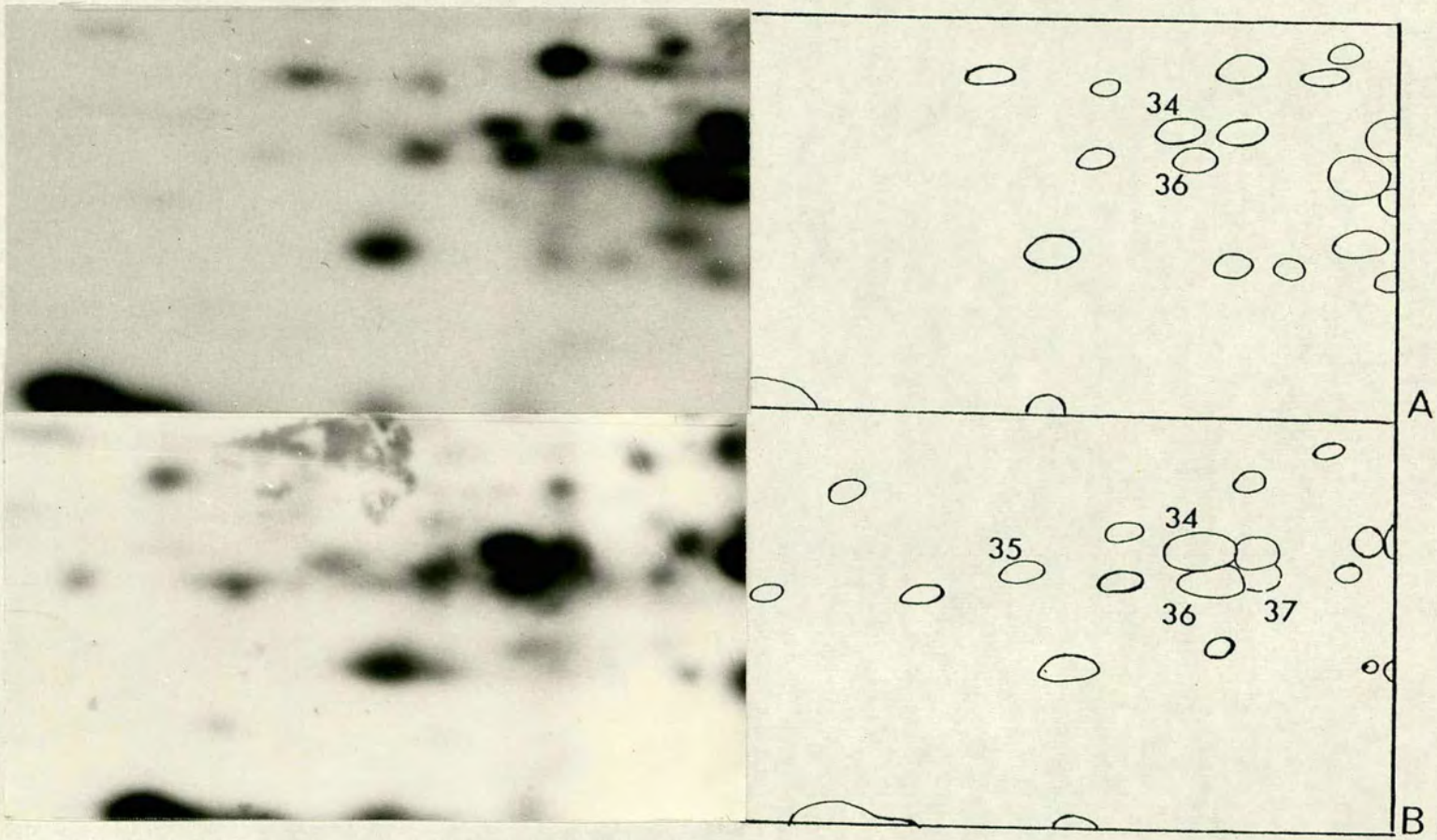


Figure 4.3

were present in 69.10C except polypeptide 15. Most of them did not show the characteristic changes observed in a/a non-sporulating or a/α sporulating cells, only polypeptides 8 and 9 showed a decrease in concentration.

Polypeptide changes in XN011 and the timing of their appearances

When resuspended in sporulation medium strain XN011 exhibited more sporulation-specific changes that did strain 69.10C (Table 4.2). Eight of the new sporulation-specific polypeptides (11, 16, 17, 20, 23, 26, 27 and 35) were observed (Figure 4.3B). All of these changes were those that appeared early, within the first 4h after resuspension into sporulation medium. Vegetative polypeptides that showed concentration increases only during sporulation of wild type a/α diploids did not show significant changes in XN011, except polypeptide 10 which showed a slight increase in concentration after 4h. Polypeptide 45 was not present (Table 4.3).

Polypeptides that appeared or showed concentration increases but that were non-specific to sporulation in the wild type showed the expected patterns of changes as seen in the a/a non-sporulating and a/α sporulating cells. The remaining seven polypeptides behaved slightly different when compared to the effect in 69.10C : 6, 8, 9 and 25 did not show significant changes, 15 disappeared after 6h, 41 was present but did not show much change and 42 was reduced in intensity after 8h (Table 4.2).

Effect of cycloheximide on polypeptide changes in 69.10C and XN011

The changes detected by two-dimensional gel electrophoresis

Table 4.2 : Nature and Timing of significant changes in polypeptides during exposure of XN011 to sporulation condition.

Nature of change	Identity of Polypeptide* and Time of First Appearance				
	2	4	6	8	16-24h
Sporulation specific appearances	(7), 17, 20	11, 16, 23	(24), (39)		(1), (2)
- NEW	26, 27, 35	(38)			
- Concentration Increase	[4], [13], [14] [37]	10, (45)		(28)	
Common appearances	29, 40, 43	12	33		
- NEW	44				
- Concentration Increase	5, 18, 19	3, 31, 32	21, 22, 30 34, 36		
Other alteration		[41]	[8], 15	[9], 42	[6], [25]

(◯) polypeptide absent, [□] polypeptide present but showed no change

* polypeptides are numbered according to the scheme given in Figure 2.7

Table 4.3 : Summary of the changes in [^{35}S]-labelled polypeptides specific to sporulation in the wild type and in the two mutants; 69.10C that is homozygous for the spo50 mutation and XN011, homozygous for the spo52 mutation.

	Time in Sporulation Medium (h)														
	WILD TYPE -5E					XN011					69.10C				
	2	4	6	8	24	2	4	6	8	24	2	4	6	8	24
Polypeptide															
New Appearances															
1					+										
2					+										
7	+	+	++	++	++										
11		+	+	+	++	+	+	+	+	+					
16		+	+	++	++		+	+	++	++		+	+	++	+
17	+	+	++	++	++	+	+	+	+	+					
20	+	+	+	+	++	+	+	+	+	+		+	+	+	+
23		+	+	+	++		+	+	+	+		+	+	+	
24			+	+	++								+	+	+
26	+	+	+	+	++	+	+	+	+	+	+	+	+	++	+
27	+	+	+	+	++	+	+	+	++	++					
35	+	+	++	++	++		+	+	++	++					
38		+	+	++	++										
Increase in concentration															
4	+	++	++	++	++										
10		+	+	++	++				+	++					
13	+	+	++	++	+										
14	+	+	++	++	+										
28				+	++	*									
37	+	+	++	++	++						*				
45		+	+	++	++	*					*				

Unless otherwise stated in the text, absence of entry indicates no changes were observed.
* These polypeptides were not detected in the mutants.

technique could be due to de novo protein synthesis or modifications of polypeptides present in the vegetative cell before sporulation was initiated. As a first step to distinguish between these two possibilities, cycloheximide was added to sporulating cultures to inhibit protein synthesis on cytoplasmic ribosomes. Eventually other selective protein synthesis inhibitors such as chloramphemicol can be used to build a complete picture of the ways in which each individual polypeptide change was accomplished.

Cellular proteins of 69.10C and XN011 were labelled continuously during presporulation growth as before. 150µg ml^{-1} of cycloheximide was added to sporulating cultures of both strains at 0, 2 and 4h after resuspension into sporulation medium. Protein extracts were prepared at 8h and 24h and subjected to two-dimensional gel electrophoresis. In both strains autofluorograms of extracts prepared at 8h and 24h did not show any difference.

Table 4.4 presents the results obtained from both strains. In 69.10C, three of the sporulation-specific polypeptides (16, 23 and 24) previously observed were completely eliminated; 20 and 26 were present. (Figure 4.5B). Polypeptides 4, 10, 13 and 14 did not show any concentration increase. The appearances of new polypeptides that were common to both a/α sporulating and a/a non-sporulating cells depended on the time at which the cycloheximide was added to the sporulating cultures. 12, 29, 40 and 43 were not present even when the inhibitor was added at 4h. 33 and 44 appeared if cycloheximide was added at 2h; 33, however was present in very low concentration. All the other non-specific polypeptides were present except polypeptides 21 and 22 (Figure 4.5B), they did not show any changes in concentration.

Four of the remaining polypeptides (8, 9, 15 and 25) were not present, 41 and 42 appeared if cycloheximide was added after 2h and polypeptide 6 was present irrespective of the time when the inhibitor was added, but it did not show any change.

Three (20, 26 and 27) of the eight new sporulation specific polypeptides observed in XNO11 were apparent after treatment with cycloheximide, 26 and 27 were however present in much reduced concentrations (Figure 4.5C). The vegetative polypeptides that increased in concentration only during sporulation of a/α diploids were all present except polypeptides 28 and 45. However, they did not show any increase in concentration. As in strain 69.10C the appearances of new polypeptides due to change in medium depended on the time at which the cycloheximide was added to the sporulating cultures. Polypeptides 29, 40 and 43 were absent regardless of the time the inhibitor was added. 33 and 44 were present if cycloheximide was added at 2h, and polypeptide 12 appeared if the inhibitor was added at 4h after resuspension into sporulation medium. Those that showed non-sporulation specific increase in concentration remained unchanged. Most of the remaining polypeptides (8, 9, 15, 25) were not present. 41 and 42 appeared if cycloheximide was added after 2h but did show any significant changes. Polypeptide 6 was present all the time, without showing any alteration.

Table 4.4 : Effect of cycloheximide on new polypeptide appearances in mutants 69.10C and XN011

	Time cycloheximide was added (h)					
	69.10C			XN011		
Polypeptides	0	2	4	0	2	4
NEW Sporulation-specific appearances						
11				-	-	-
16	-	-	-	-	-	-
17				-	-	-
20	+	+	+	+	+	+
23	-	-	-	-	-	-
24	-	-	-			
26	+	+	+	+	+	+
27				+	+	+
35				-	-	-
NEW Common appearances						
12	-	-	-	-	-	+
29	-	-	-	-	-	-
33	-	+	+	-	+	+
40	-	-	-	-	-	-
43	-	-	-	-	-	-
44	-	+	+	-	+	+

Cycloheximide was added at 0, 2, 4h after resuspension into sporulation medium and protein extracts were prepared after 8h. All vegetative polypeptides that increased in concentration during sporulation of the a/α wild type diploid (if present) did not show any concentration increase in 69.10C and XN011.

\+ indicates appearance of polypeptide

\- indicates polypeptide would have appeared in the absence of cycloheximide absence of entry indicates polypeptide was not observed in mutant

Figure 4.4 : Sections of autofluorograms of [^{35}S] - labelled polypeptides showing the effect of cycloheximide on the presence of polypeptides 34 to 37. (A) 69.10C and (B) XN011 after 8h in sporulation medium, cycloheximide was added immediately after resuspension. Polypeptide 35 was not present in either strain.

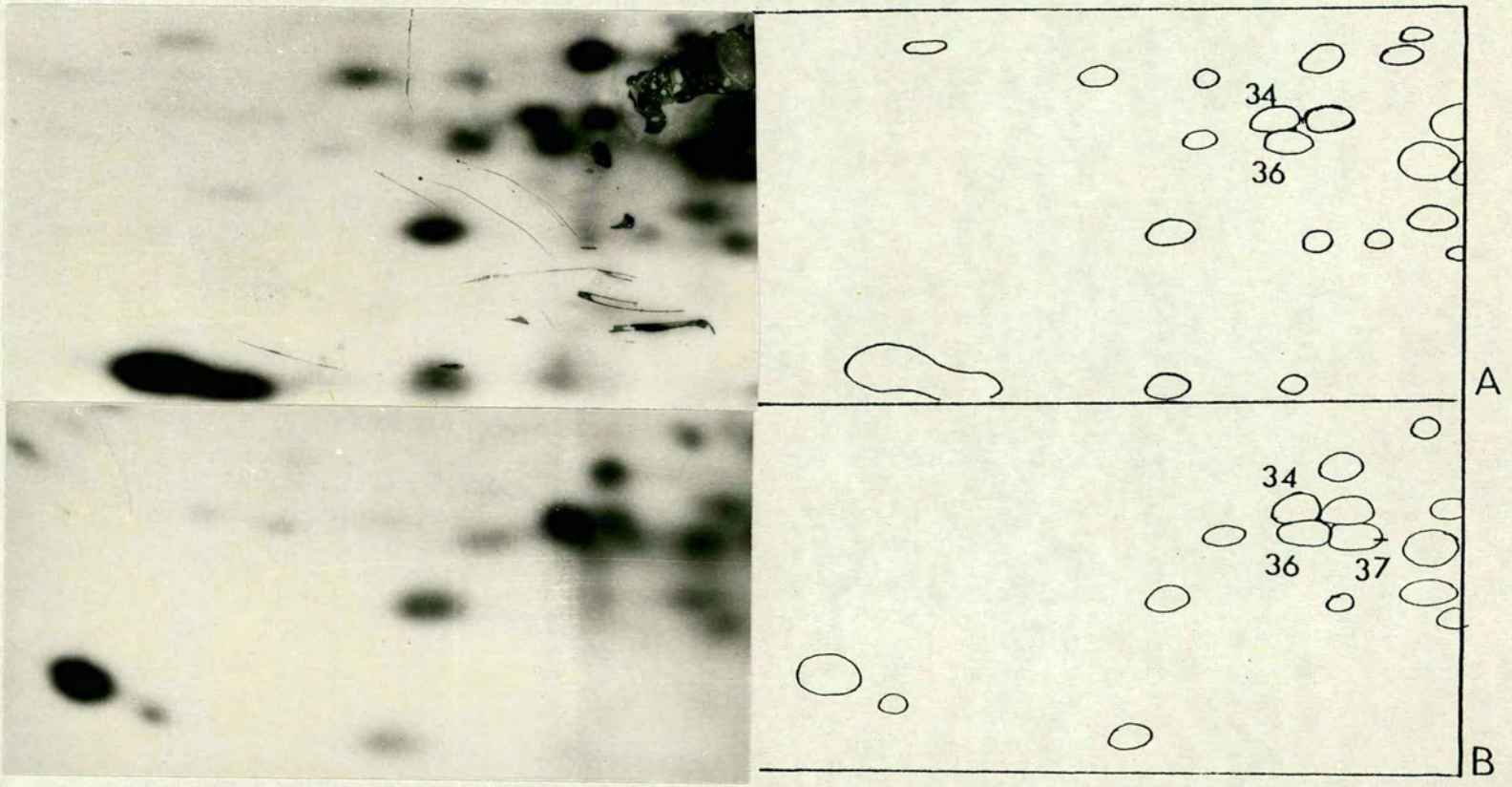


Figure 4.4

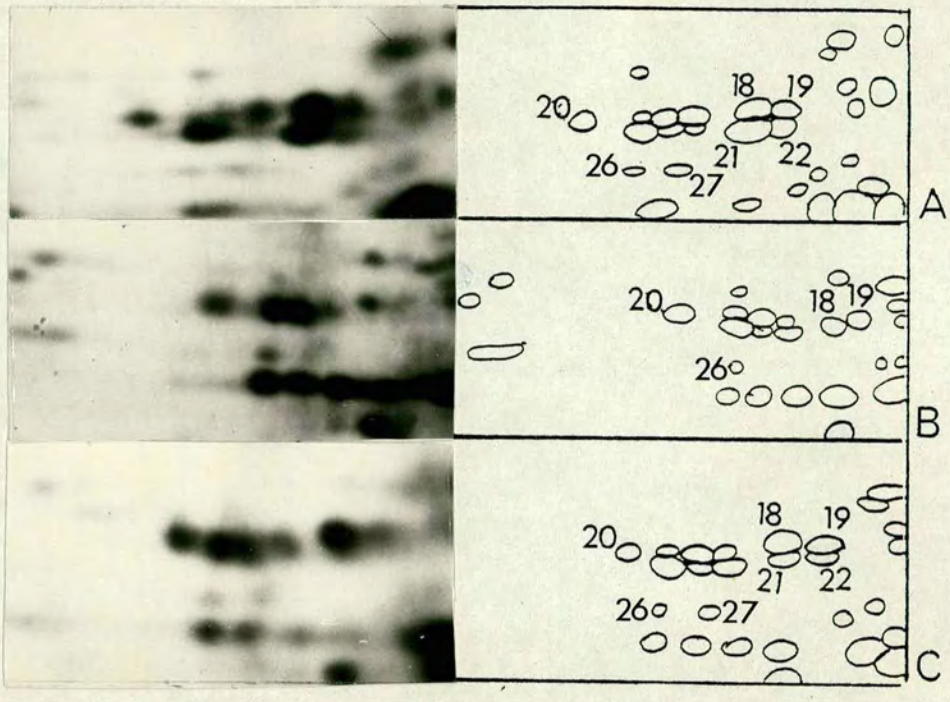


Figure 4.5 : Sections of autofluorograms showing the effect of cycloheximide on polypeptides 18, 19, 20, 21, 22, 26 and 27 (A) a/α diploids 24h in sporulation medium without cycloheximide (B) 69.10C & (C) XN011, cycloheximide added immediately after resuspension into sporulation medium. Protein extracts were prepared from cells 8h after resuspending them in sporulation medium.

Effect of cycloheximide on sporulating cultures

Cycloheximide blocks cytoplasmic protein synthesis in eukaryotes by inhibiting translocation steps (in much the same way that chloramphenicol acts on prokaryotic protein synthesis). The action of the inhibitor is normally reversible (treated cells resume growth if transferred to fresh medium), but it may be that under starvation conditions cells lose viability dramatically in the presence of the drug and that its effect on sporulation is due more to irreversible cell damage than to inhibition of protein synthesis that is essential to sporulation. This was tested by examining the effect of cycloheximide on viability of the a/α sporulating wild type and the two mutants, 69.10C and XN129 during resuspension in sporulation medium.

Exponentially growing populations of 135.11B (a/α sporulating strain), 69.10C and XN011 were resuspended into sporulation medium and cycloheximide was added immediately. Samples were taken at 2h intervals from all cultures and appropriately diluted, plated on YEPD and incubated at 30°C. Survivors scored after 3d growth indicated that colony forming ability of all strains was reduced to about 60% (Figure 4.6). Viability of cells of strain 69.10C, which have been shown to be low compared with that of other strains (Figure 3.3), was not aggravated significantly by the addition of cycloheximide.

Microscopic examination of the populations after 24h resuspension in sporulation medium treated with cycloheximide showed that no asci had formed in the a/α sporulating diploid, a high proportion of the cell population of all strains remained in the form of buds attached

to mother cells, i.e. the cells were not able to complete the mitotic cycle and enter the G1 phase as usually happens when cells are exposed to limiting nutrient conditions (Hartwell, 1974).

The above results indicate that cycloheximide treatment does cause a slow and gradual decline in viability of cells under starvation conditions, but there is nothing severe enough to consider that its use in studying the requirement for protein synthesis during sporulation is not warranted. It should be noted that it also arrests cells in the cell division cycle, and since sporulation can only be initiated at the G1 phase, it may prevent sporulation if added at zero time by preventing progression of the cells through the mitotic cell cycle. Other workers have however shown that it inhibits ascus formation when added at any time until just before asci would normally appear (Magee & Hopper, 1974).

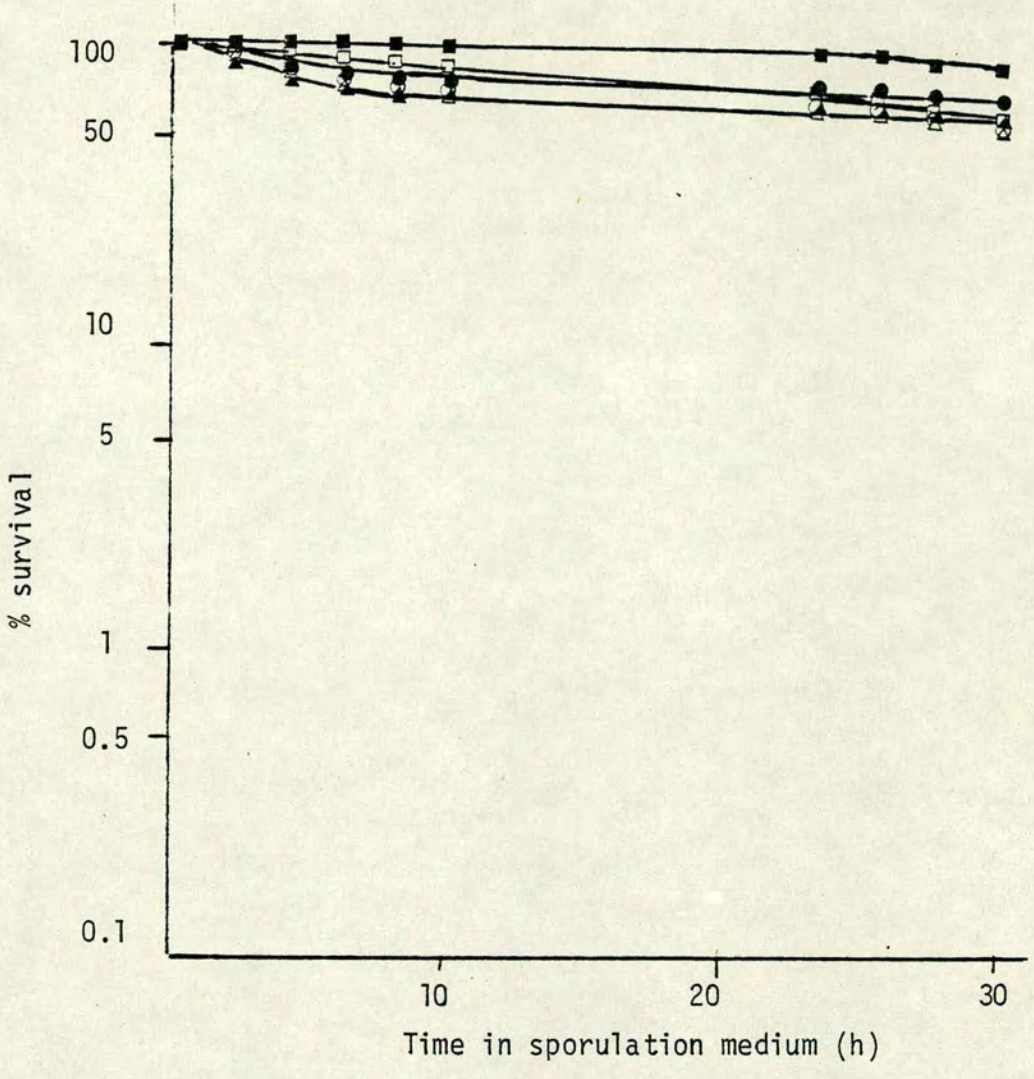


Figure 4.6 : Survival of the wild type and mutants 69.10C and XN129 in sporulation medium with cycloheximide added immediately after resuspension. (□) 135.11B, (△) 69.10C, (○) XN129, (■) (▲) & (●) were survival of the respective strains in sporulation medium without cycloheximide.

Discussion

The polypeptide changes observed in both mutants differentiated them from strains that are unable to sporulate due to homozygosity (a/a and α/α) at the mating-type locus (Roth & Lusnak 1970). The presence of the a/α alleles allowed the appearance of several sporulation-specific polypeptides, even in strain 69.10C which did not show any other detectable event that has previously been shown to accompany sporulation. The result with the supposed initiation mutant 69.10C indicated that the mating-type locus control over sporulation is probably acting very early in the process, and it confirms that the use of a/a and α/α diploids as controls in the study of sporulation-specific events has some validity.

All new polypeptide appearances and alterations specific to sporulation that occurred in 69.10C and XN011 were those that appeared early in the a/α sporulating diploid. None of the late changes were observed. This is consistent with the view that later sporulation events are dependent on successful completion of most (if not all) earlier ones.

The other polypeptide changes i.e. those that were non-specific to sporulation, confirmed earlier results. They were presumably brought about by the cells adapting to the change in medium. It is also clear, on considering what happened in 69.10C to the non-specific (starvation-induced) changes that occurred in both a/a and a/α wild type strains, that after 6 to 8h resuspension in sporulation medium the mutant was no longer capable of responding to the starvation

conditions by altering its protein complement in the same way as the wild type. Thus it would appear that not only does the spo 50 mutation block sporulation very early, it also leads to aberrant behaviour in response to starvation. This is consistent with the finding that strain 69.10C loses viability if left in sporulation media for extended periods (also G.R. Calvert, personal communication). This could be taken to indicate that non-sporulating cells of wild type strains undergo a 'programme' of protein changes following the onset of starvation, and that this programme is also affected by the spo 50 mutation. Another possibility is that in 69.10C the cells by virtue of trying to sporulate in the absence of an essential function undergo an abnormal series of changes resulting in loss of viability.

Although strain 69.10C was blocked very early, it was able to exhibit five sporulation-specific polypeptides changes. They were probably involved in initiation of sporulation, the mutant was able to respond to the inductive conditions allowing synthesis or modifications of pre-existing polypeptides synthesised during the vegetative growth but due to a defect imposed by spo 50 further development was inhibited.

Another interesting observation is that three of the vegetative polypeptides that later increased in concentration during sporulation of a/α diploids, were not observed in strain 69.10C and two were missing in XNO11. These polypeptides could be due to differences in the strains as illustrated by polypeptide 45. It has been shown that this polypeptide was associated with the spd1 gene.

XN011 did have this mutation. However early blocked sporulation mutants derived by reversion of spd 1 strains such as 69.10C do not synthesise the protein (Dawes & Martin, 1981). The other polypeptides could be directly involved in sporulation. Since the a/a strain has no other sporulation defect than to be blocked by the mating-type locus, synthesis occurred at a low level during vegetative growth just as in a/α diploids. Sporulation created demand for the polypeptides as reflected by their concentration which increased in the a/α sporulating cells only. In the two mutants, synthesis was blocked even during vegetative growth, however XN011 was blocked later than 69.10C therefore was able to synthesise more polypeptide.

Continuous protein synthesis has been shown to be required for ascospore formation until immediately before the appearance of ascospores (Esposito et al., 1969; Magee & Hopper, 1974). Adaptation of yeast cell populations to low levels of nutrients appears to have the same requirement. Analysis of protein extracts prepared from cycloheximide-treated sporulating cultures indicated that not only most of the sporulation-specific appearances and alterations were eliminated, but that many of the changes that were not specific to sporulation were also not observed. These changes appeared to be well coordinated since later addition of cycloheximide allowed the appearance of several polypeptides that have been shown to be involved in adaptation to the starvation conditions.

Three sporulation-specific polypeptides in XN011 and two in 69.10C were resistant to the inhibitory effect of cycloheximide.

These polypeptides could be due to modifications or to mitochondrial protein synthesis. Analysis of polypeptide complements from cultures of the same strain treated with chloramphenicol (which inhibits mitochondrial protein synthesis) and comparison with polypeptide patterns from cycloheximide treated sporulating cultures as has been done in this project should be able to differentiate between the two possibilities. For example a change that is resistant to both cycloheximide and chloram phenicol would therefore be probably due to modification and the enzyme(s) causing the modification was itself present and did not need to be synthesised. On the other hand cycloheximide-sensitive changes could still involve modifications (i.e. the spot may be 'new' due to a modification involving an enzyme that has to be synthesised de novo) but such changes do imply that nuclear gene expression is needed, and many of these changes may be due to de novo protein synthesis. Changes that are resistant to cycloheximide but sensitive to chloroamphenicol would indicate de novo synthesis or modifications in mitochondria . Isolating mitochondria at various intervals during sporulation; preparing protein extracts and subsequent two-dimensional gel electrophoresis would give results which could be compared with results from cycloheximide and chloramphenicol treated cultures. These would further confirm whether the involvement of proteins synthesised in the mitochondria is part of the programme of spore formation.

Further analysis of protein extracts involving treatment with chloramphenicol was not carried out, since two-dimensional gel electrophoresis requires a lot of time and this commodity was no longer available for this project.

It must also be noted that the above results on the effect of cycloheximide on sporulation-specific polypeptide appearances were obtained from experiments done with the two asporogenous mutants. A much more detailed analysis using the wild-type and more time points needs to be done to obtain a more complete picture of the dependence of the various changes on protein synthesis. Attempts to do this were made, but were hampered by difficulties in maintaining and handling the wild type strains used in the project, and by the time (or lack of it) available.

SECTION V

N-METHYL-N'-NITRO-N-NITROSOGUANIDINE MUTAGENESIS DURING MEIOSIS AND SPORE FORMATION

It has been suggested that N-methyl-N'-nitro-N- nitrosoguanidine (NTG) mutates nuclear genes in yeast as they are replicating (Burke & Fangman, 1975; Dawes & Carter, 1974; Kee & Haber, 1975). DNA replication is slower in cells undergoing sporulation (Williamson et al., 1980) and it has been possible to synchronise yeast sporulation to a reasonable extent (Fast, 1973; Roth & Halvorson, 1969). Thus it may be possible to determine whether during premeiotic DNA synthesis there is a similar enhancement of mutation by NTG and, if so, if there is any pattern of replication of any particular gene.

Another reason that studying mutation by NTG during sporulation might be useful comes from the report that some mutagens, including NTG, may act preferentially during transcription of certain genes (Brock, 1971; Savic & Kanazir, 1972). While NTG does appear to enhance mutation rates in vegetative cells that are actively replicating their DNA, it does also act on non-replicating cells to a lesser extent. It may therefore be the case that NTG treatment of sporulating cells could provide an enhancement of mutation at those loci that are important to sporulation. This possibility was tested briefly.

Preliminary screening for markers suitable for NTG induced mutation

Haploid strains of Saccharomyces cerevisiae bearing several auxotrophic markers were tested for their responses to NTG using the 'ring'-test. A minute crystal of NTG, was placed in the centre of a YEPD plate covered with a lawn of the strain to be tested. The NTG was absorbed and diffused through the agar forming a radial gradient. It killed the cells until an area of sublethal concentration was reached, in which it induced reversion of those auxotrophic markers that were susceptible. When the plate was replica plated onto complete medium lacking the auxotrophic requirement, the reverted cells grew in a ring. Not all markers were reverted by NTG (Table 5.1) the his 1 marker in Y174 and the trp 1 marker in Y323 gave the best responses (Figures 5.1 and 5.2).

Y174 and Y323 were crossed to a haploid progeny of a sporulation competent heterothallic diploid. Asci from the resulting diploids were dissected and a and α spores carrying his 1 or trp 1 markers were crossed to each other. Sporulation of the resulting heterozygous diploids allowed the selection of haploid spore progeny carrying both mutations. They were intercrossed and screened for good sporulation ability. A diploid 14A x 17C (a/α his1/his1 trp1/trp1 ade1/ADE1 was selected for further experiments.

Response of vegetatively growing 14A x 17C to various concentration of NTG

In order to test the response of the markers in 14A x 17C, samples from logarithmic phase cultures of the diploid were treated

Table 5.1 : Response of markers in three strains of haploid Saccharomyces cerevisiae to NTG treatment (ring test)

MARKERS \ STRAIN	Y174	Y323	A364A
ADE	0	0	0
ARG	0	-	-
HIS	+	0	0
LEU	-	+	-
LYS	-	-	0
MET	-	0	-
THR	0	-	0
TRP	0	+	-
TYR	-	-	0
URA	0	+ -	0

Y174: α ura3 thr3 his1 arg6 trp2 ade1

Y323: α ade1 gal1 trp1 ura3 his2 leu1 met14

A364A: a ade1 ade2 ura1 his7 lys2 tyr1 gal1

(0) no reversion (+) reversion (-) marker not present

Figure 5.1 : Photograph of test plate showing NTG induced reversion at the *his1* locus of strain Y174. Strain Y174 was spread on YEPD and a minute crystal of NTG was placed in the centre, the plate was incubated at 30°C for 3d then replica plated onto complete medium lacking histidine. Further incubation allowed reverted cells induced by sublethal concentration of NTG to form colonies.

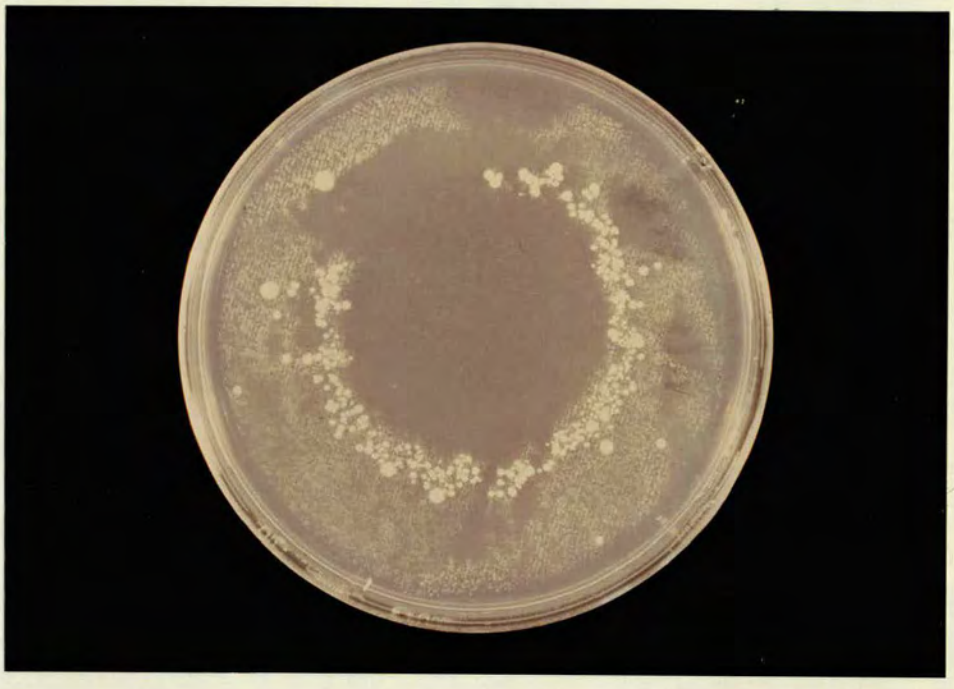


Figure 5.2 : Photograph of test plate showing NTG induced reversion at the trp1 locus of strain Y323 on complete medium lacking tryptophan. (The technique used was similar to that for strain Y174 [Figure 5.1])

with increasing concentrations of NTG in liquid medium, diluted appropriately for plating. After 3d growth on YEPD at 30°C the number of survivors was counted. Revertants were counted by replicating on complete medium minus histidine or tryptophan Maximum revertants were obtained when samples from the culture were treated with 500μg ml^{-1} of NTG. The viability of the population was reduced by 60% at this dose (Figure 5.3).

Effect of NTG at various stages of sporulation of 14A x 17C

As indicated in the experiment with the vegetative population, 500 μg ml^{-1} NTG treatment gave the maximum level of revertants. This concentration was used to induce mutation in sporulating cultures at hourly intervals. Figure 5.4 illustrates the effect of NTG on the survival of the population during sporulation. While the untreated sporulating population remained more or less at the same viable cell concentration, the treated population was adversely affected between 3 to 6 h. This period corresponded to the time at which the rate of DNA synthesis in 14A x 17C was maximal (Figure 5.5).

The trp1 and his1 markers reverted independently showing two distinct peaks of susceptibility as shown in Figure 5.6. The trp1 revertants reached a maximum at 5 h after resuspension into sporulation medium, about an hour after the his1 revertants, the latter however did not reach the same proportion as the trp1 revertants. The second peaks for both markers more or less coincided at about 8h but were less significant compared to the earlier ones. These peaks probably corresponded to the second meiotic division, at which the

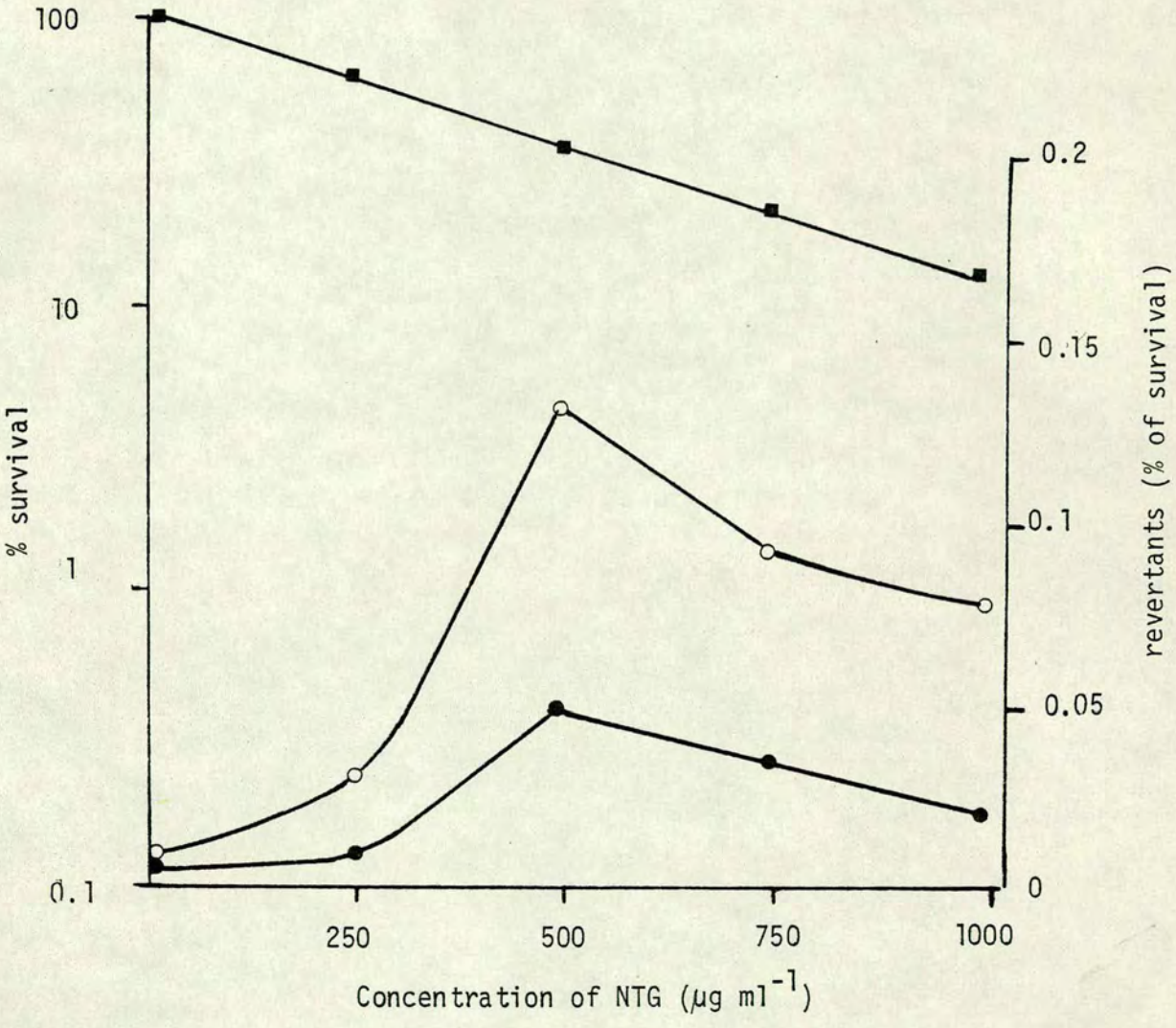


Figure 5.3 : Survival of vegetative 14A x 17C in different concentrations of NTG, and the percentage of revertants induced. (■) % survival (○) TRP^+ revertants (●) HIS^+ revertants. Cell samples were treated with NTG for 10 min at 30°C, diluted and plated on YEPD for estimation of the number of survivors (after 3d. incubation at 30°C). Revertants were counted by replicating the survivors on complete medium minus histidine or tryptophan and further growth at 30°C for 2d.

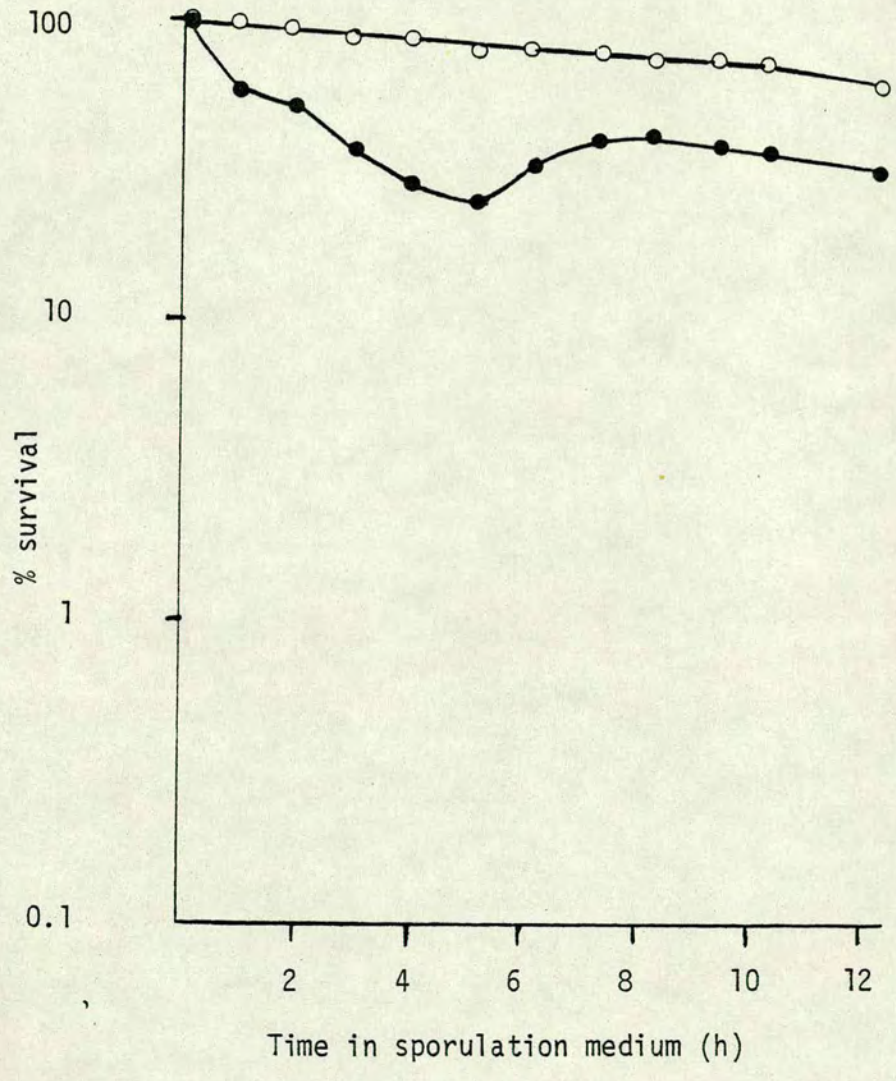


Figure 5.4 : Effect of NTG on sporulating population of 14A x 17C
Samples were taken at hourly interval and treated with 500µg ml^{-1} NTG for 10 min at 30°C, diluted and plated on YEPD. Survivors were scored after 5d growth at 30°C (○) untreated population (●) treated population.

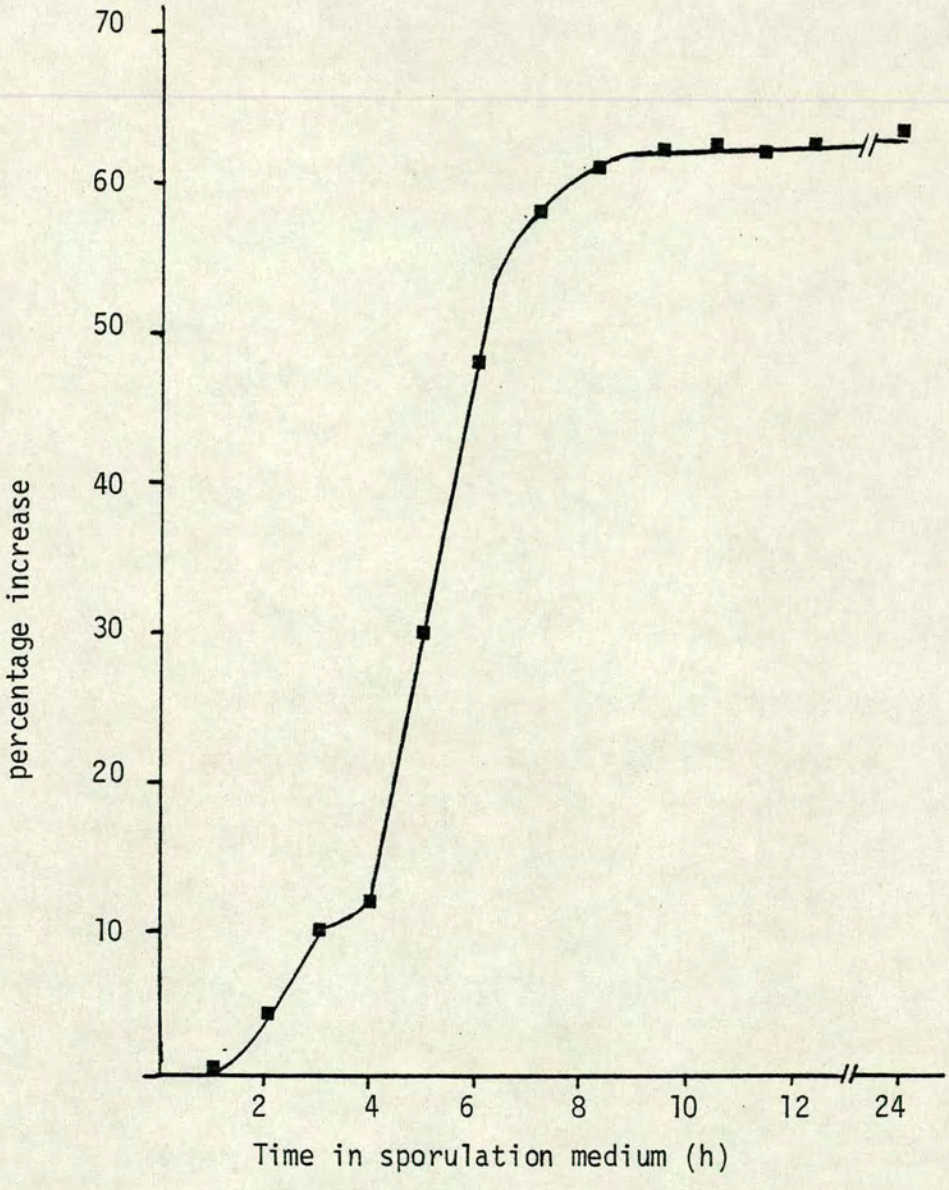


Figure 5.5 : DNA synthesis during sporulation of 14A x 17C. Duplicate samples were taken at hourly intervals and DNA was assayed according to the method of Stewart (1975). The first ascus was observed 14h after resuspension in sporulation medium. By 24h 70% of the population had formed asci.

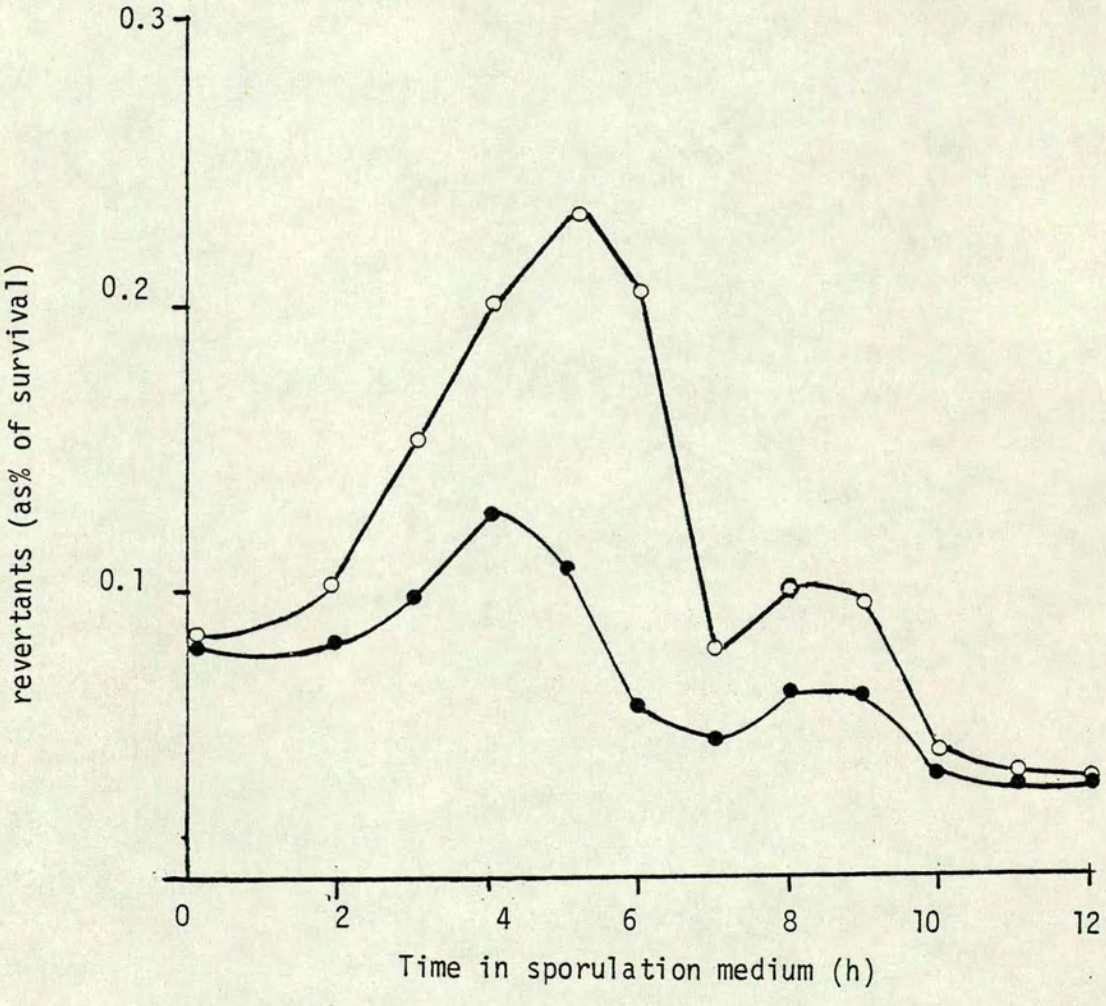


Figure 5.6 : Revertants after treatment with NTG (500µg ml^{-1}). After growth on YEPD (Figure 5.4) survivors were replica plated onto complete medium minus histidine or tryptophan. Revertants were scored after 3d growth at 30°C (○) TRP^+ revertants (●) HIS^+ revertants.

the separation of the chromosomes increased the susceptibility of the DNA to NTG, but not to the same extent as during DNA replication.

Genetic analysis of revertants

Reversion of the auxotrophic markers could be due to mutation at the original sites of the genes or to mutation in unlinked suppressors. In the former case, the peak of revertants would probably indicate the time of replication of the marker gene and the latter, represent the time when the suppressor locus was duplicated. Since the strain (14A x 17C) was a diploid, the revertants could either be heterozygous (A/a) or less likely homozygous (A/A) for reversion of the auxotrophic markers or the auxotrophic markers would have remained in their recessive state with a dominant mutation at a suppressor locus affecting their expression (a/a SUP/SUP; a/a SUP/sup).

In order to test all these possibilities, reverted 14A x 17C was sporulated and asci dissected for tetrad analysis. Both markers showed a 1:1 segregation of prototrophic to auxotrophic spore indicating the mutations could either be A/a or a/a SUP/sup. If it was the latter, a cross between the prototrophic spores with a wild type haploid should give 25% auxotrophic spores. Such crosses for his1 revertants progeny gave all histidine-independent spores (Table 5.2), thus the reversion was due to mutation at the site of the original marker, mutating one allele to give the heterozygous dominant HIS/his. Crosses with the tryptophan-independent spores yielded 25%. (ranged between 18-31%) tryptophan-dependent spores, indicating that reversions were largely due to a mutation at a suppressor locus.

Table 5.2 : Genetic analysis of NTG induced revertants

	Spore genotype	
Revertant type	100% Prototrophic	75% Prototrophic
His^+	$12/12^+$	0/12
Trp^+	$1/15^+$	14/15

Trp^+ and His^+ spores from 14AX17C induced by NTG were crossed to wild type haploids. Spores from the resulting diploids were subjected to random spore analysis. ($^+$ number of diploids tested).

Tryptophan and histidine-independent spores from reverted 14A x 17C were further tested by crossing to a haploid strain bearing arg 4-17, his 5-2 and lys 1-1 markers suppressible by ochre suppressors and an amber suppressible trp 1-1 marker. Table 5.3 summarises a typical result of tetrad analysis of such crosses. All the markers yielded 1:1 ratio of prototrophs to auxotrophs, including the trp markers which did not complement each other. These results confirmed that reversion in the marker in 14A x 17C was due to a dominant mutation in a suppressor locus, probably to an amber-specific suppressor which does affect the trp1-1 locus (Mortimer & Hawthone, 1969). The trp1 allele from Y323 is therefore probably the result of an amber mutation.

Although the mutation in the suppressor gene was able to alleviate the tryptophan requirement, it was not as efficient as reversion at actual marker genes. On supplemented minimal plates lacking tryptophan, most of the tryptophan independent spores derived from NTG-treated 14A x 17C showed slightly reduced growth compared to the wild type.

Diploids were constructed from trp-independent spores of NTG treated 14A x 17C to see if the suppressor gene had any effect on sporulation. In both homozygous and heterozygous states of the trp marker, there was no reduction in sporulation ability compared to the original 14A x 17C.

Induction of sporulation mutants by NTG

The main aim of the experiment was to examine the effect of NTG on "sporulation-specific" genes involved in meiosis and sporulation

Table 5.3 : Suppressor of trpl mutation. The cross a/α arg4-17^{o}/ARG4-17 his5-2^{o}/HIS5-2 lys1-1^{o}/LYS1-1 trp1-1^{a}/trp1-x was dissected to test whether the suppressor was an amber or an ochre suppressor.

	Tetrad type		
	4.0	3.1	2.2
arg4	0	0	100
his5	0	0	100
lys1	0	0	100
trp1	0	0	100*

o markers suppressible by ochre-specific suppressor

a markers suppressible by amber-specific suppressor

*2.2 asci only expected from a trp1-1/trp1-x^{+}/sup (+ indicated wild type allele of SUP gene) if both trp1 alleles are suppressible by the same SUP gene. Therefore, since trp1-1 is amber suppressible, trp1-x is also probably an amber suppressible allele and SUP gene is an amber specific.

(Results given were as percentage of total asci)

and to determine whether there was any pattern of replication of the genes, or possibly of their transcription.

The survivors after treatment with NTG at different time points were therefore exposed to sporulation conditions and screened for their ability to sporulate. Colonies on potassium acetate plates were replica-plated onto YEPD in glass petri dishes and these were exposed to diethyl ether vapour for 90 min at 30°C in a sealed vessel. The plates were incubated at 30°C for 3-5d. Those colonies that were not able to complete sporulation showed poor growth or were not able to grow; they were more sensitive to the ether vapour compared to asci. These colonies were further screened by microscopic examination.

The effect of NTG on the sporulating ability of the population varied according to the time the cells were exposed to NTG. Treatment between 3 and 8h gave a percentage of cells unable to complete sporulation, whereas later treatment did not seem to give any mutants of this type. The results again indicated the susceptibility of the strain to NTG during meiotic DNA synthesis. However inability to sporulate does not necessarily arise due to mutations in sporulation-specific genes, most of the cells that were unable to sporulate were petites as shown by their inability to grow on glycerol-containing medium. Mutations which resulted in petites are easily induced by most mutagens. Petites have lost the ability to oxidise substrates via the TCA cycle, which is an absolute requirement of sporulation (Kuenzi et al., 1973; Pratje et al., 1979).

The above experiments were done using a heterothallic diploid

14A x 17C which was purposely built with markers susceptible to NTG to facilitate observations on the effect of the mutagen on cells undergoing meiotic DNA replication. Any recessive sporulation mutation induced on a single copy therefore would not be expressed in the treated diploids and would not be detected in their progeny because most sporulation gene can only be expressed in diploid strains.

A homothallic strain, S41 was sporulated and cell samples were treated with NTG at intervals, diluted and plated on YEPD plates. After 3d incubation at 30°C the plates were replica plated on potassium acetate and screened for sporulation ability to detect dominant mutations. Since there were large numbers of sporulating colonies from each time point, it was not possible to do the subsequent screening on all of them. As many sporulating colonies as possible were treated with glusulase to release the ascospores, they were diluted and plated on YEPD plates such that the density on each plate was low and colonies well-spread. Since they carry the homothallism gene, outgrowth should have resulted in the formation of homozygous diploids in which any recessive mutation could be expressed. The colonies formed were replica plated onto potassium acetate to allow sporulation, then onto YEPD in glass petri dishes and treated with ether vapour. Colonies that were unable to sporulate were sensitive to the ether and thus showed no growth after subsequent incubation. Such colonies were rare and some of the few isolated were petites, probably resulting from mutations in nuclear genes. There was no indication of enhanced induction of mutations to asporogeny at any time point. In general terms, there were too few mutants obtained to relate the result to the time of replication or transcription of sporulation-specific genes during meiotic DNA synthesis or at other times during sporulation.

Discussion

The response of the two markers to NTG mutagenesis during meiotic DNA synthesis agreed well with the view that NTG preferentially mutates during DNA replication. The main peaks of reversions of the two markers tested coincided with the period of rapid DNA synthesis in the strain. The enhanced reversion of the auxotrophic markers at different times during meiotic DNA synthesis presumably reflects the time at which replication of the marker genes (or replication of the tRNA genes responsible for the suppressor mutation). The observation that individual genes gave maximum response to NTG at different time suggested that the replication of DNA during meiosis is well controlled, involving definite start points that are activated at different times during the process. There was also an increase in sensitivity of the cells to the lethal effects of NTG during active meiotic DNA synthesis; possibly strand separation makes the nucleotide bases more accessible to the mutagen which not only enhanced mutation frequency but also increased the killing rate. The second peaks of reversion of the markers probably coincided with the time of second meiotic division in which chromosome segregation may have made the DNA vulnerable but not as exposed as during replication, thus the effect of NTG was not as severe.

It was speculated that genes being transcribed during meiosis might show a similar response and since genes directly involved in sporulation are probably only activated during the process, NTG mutagenesis of samples taken at intervals during sporulation may enhance mutation at these loci. This however was not observed, the

number of mutants obtained was not significantly higher than that expected for induction by other mutagens and there was no indication of increased susceptibility at any time during the process.

Unlike some Bacillus spp. which produce pigments giving a characteristic brownish colour to sporulating colonies (Schaeffer, 1969), yeast do not have any indicative appearance when undergoing sporulation. Screening for sporulation mutants which has to be done on large number of colonies is carried out by microscopic examination, which is not only tedious and time consuming but has its limitations. The diethyl ether technique has tremendously assisted in differentiating sporulating and vegetative cultures. However a second microscopic screening was sometimes necessary to ensure the strains were positively asporogenous. The limitations of screening technique could contribute to the result obtained.

Another factor which can affect the efficiency of isolation of sporulation mutants is ploidy : recessive mutations induced in a single copy of the DNA are not expressed in a diploid. When mutated sporulation genes segregate during spore formation, detection is again impossible (since haploids do not sporulate) unless it concurrently affected other recognisable features such as are known for the spd1 mutation which showed poor growth on glycerol (Vezinhet et al., 1979). An alternative which enables the detection of recessive mutations induced in diploids is the use of homothallic diploid strains. As discussed in the Introduction of this thesis, the homothallic gene induces self-diploidisation of haploid ascospores, thus recessive mutations segregated during meiosis are expressed. Even with this technique, enhancement of mutations in genes that affected sporulation after NTG mutagenesis was not detected.

SECTION VI

DEVELOPMENT OF A CLONING SYSTEM TO ISOLATE SPORULATION SPECIFIC GENES IN SACCHAROMYCES CEREVISIAE

Sporulation involves a series of morphologically distinct stages which result in the formation of mature ascospores within an ascus. Various biochemical events occur during these stages of spore formation and, as shown in Section II, polypeptide changes are involved and these appear in a temporally well-defined sequence. This developmental programme in part probably reflects an ordered progression in the transcriptional activation of sporulation genes. So far, however, it has not been possible to show detailed changes in the pattern of transcription of these genes during the course of sporulation development. In fact when the work on this thesis was begun it was not even clear whether there were any genes specifically transcribed during yeast sporulation.

Recently, several techniques for the cloning and isolation of specific genes have been developed in yeast (Beggs, 1978; Broach, et al., 1979; Gerbaud et al., 1979; Hinnen et al., 1978). With the development of methods for transforming yeast, it is now feasible to clone genes that are specifically concerned with sporulation by complementing defects in asporogenous mutants by the introduction of plasmid-borne wild-type DNA. Two quite well characterised asporogenous mutants were already available. These provide an opportunity to explore the possibility of using these techniques to isolate sporulation-specific genes in these two mutants and to study the regulatory mechanisms involved.

Propagation and amplification of plasmid DNA

A hybrid plasmid, pJDB207 (obtained from Dr. M. Smith, Molecular Biology, Edinburgh) was employed; the physical map is shown in Figure 6.1. The plasmid carries the yeast *LEU2* gene which codes for β-isopropylmalate dehydrogenase in yeast and ampicillin and tetracycline resistance genes (from the bacterial plasmid pBR 322) that are expressed in *E. coli*.

E. coli strain HB101 was used to propagate the plasmid. HB101 was made competent by calcium chloride addition and was transformed by heat shock treatment with purified plasmid DNA as described by Beggs (1978). Transformants were selected on L-broth plates containing ampicillin (100 $\mu g\ ml^{-1}$) and plasmid DNA was prepared from transformed clones. To amplify the yield of plasmid DNA, cultures of transformed HB101 were treated overnight with chloramphenicol (150 $\mu g\ ml^{-1}$) before plasmid DNA was prepared. Chloramphenicol allowed the replication of plasmid DNA, whereas for chromosomal DNA only the round of replication in progress was able to complete without further rounds initiated (Clewell, 1972). Figure 6.2 illustrates the results obtained from amplified and non-amplified cultures after the plasmid DNA was isolated from them and electrophoresed on a 1% (w/v) agarose gel.

Transformation of strain A22 with plasmid pJDB207

A haploid yeast strain AH22 bearing 2 alleles of the *leu2* gene (*a leu 2-3 leu 2-112 his 4 can 1*) was used as the recipient strain to investigate the uptake of plasmid pJDB207 and the transfer of the LEU^+ phenotype. Yeast protoplasts were prepared according to the method

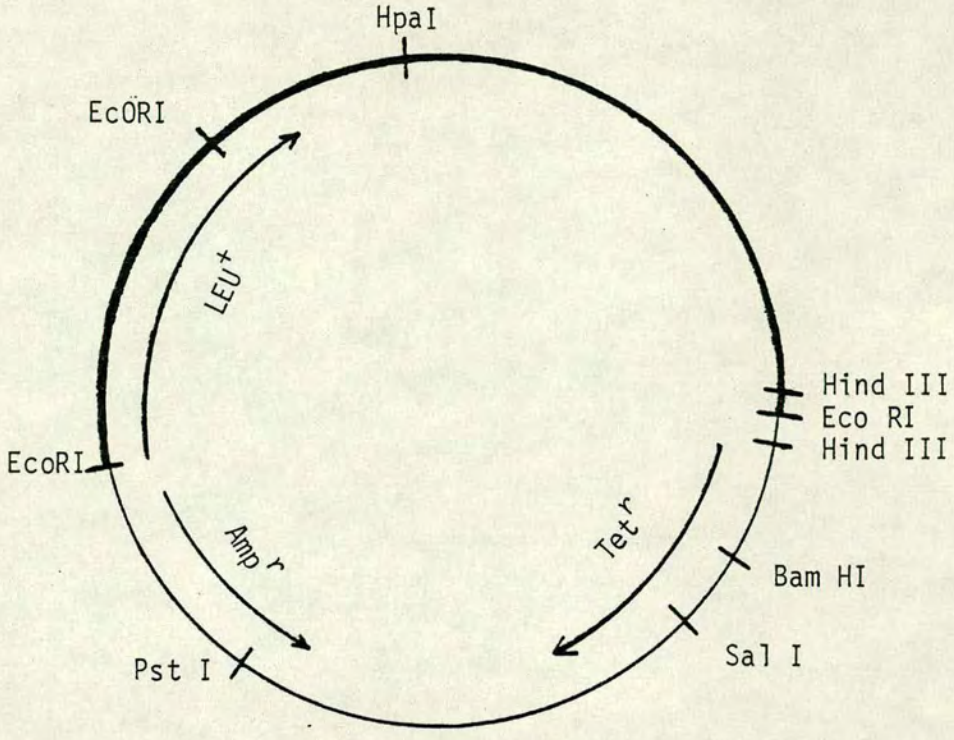


Figure 6.1 : Schematic drawing of plasmid pJDB207. This was built from plasmids pBR322 (thin line) and pYleu10, a ColE1 derivative (carrying the complete LEU2 gene of yeast; thick line). The location of restriction sites on plasmid pJDB207 was determined by single and multiple restriction digestion of the purified plasmids. Fragment sizes were determined by comparison of migration distances with those of pBR322 restriction DNA fragments of known size after electrophoresis on agarose gels. This plasmid was constructed and characterised by Beggs (1978).

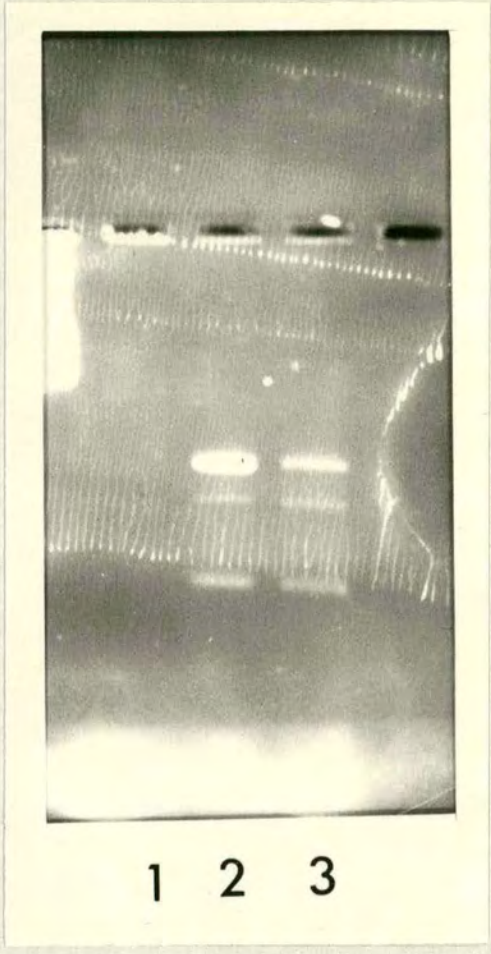


Figure 6.2 : DNA prepared from HB101 and pJDB207 - transformed E. coli strains, electrophoresed on a 1% (w/v) agarose gel stained with ethidium bromide. The sources of DNA were : (1) HB101 parent strain, (2) Transformed HB101 amplified overnight with chloramphenicol (3) Unamplified Hb101 transformed with pJDB207.

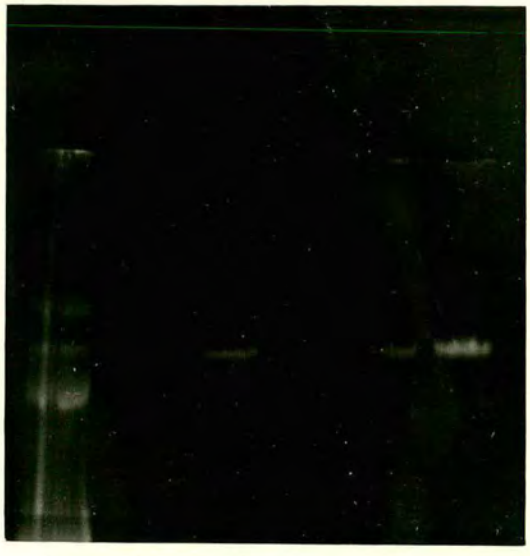


Figure 6.3 : DNA prepared from the S. cerevisiae AH22 transformed to LEU^+ with pJDB207 from the AH22 parent strain and pJDB207; electrophoresed on a 1% (w/v) vertical agarose gel and stained with ethidium bromide. The sources of DNA were: (1) pJDB207 prepared from transformed E. coli strain HB101 (2) Untransformed AH22, (3), (4) & (5) strains of AH22 transformed to LEU^+ with pJDB207.

of Beggs (1978). Protoplasts and purified plasmid DNA were mixed and treated with polyethyleneglycol to promote DNA uptake. Control experiments using treated protoplasts plated on supplemented minimal medium indicated that 20% of the total protoplasts formed were able to regenerate cell wall and grow into colonies. Samples plated on supplemented minimal agar lacking leucine incubated for 5-7 days to allow colonies to develop showed 1 in 10^5 yeast protoplasts were able to take up the plasmid with complementation of the leu2 gene in AH22. Figure 6.3 illustrates the result after electrophoresis on a 1% agarose gel of plasmid DNA prepared from transformed yeast clones of AH22. A distinct band was exhibited by DNA prepared from each transformant, and this band was not observed in an untransformed AH22 culture similarly treated.

Transfer of leu 2 markers into asporogenous mutants

The presence of the LEU2 yeast gene in pJDB207 makes this plasmid a suitable cloning vector for isolating sporulation genes. Random yeast DNA fragments generated by restriction with Bam HI endonuclease can be inserted into the Bam HI site of the plasmid, inactivating the gene conferring tetracycline resistance. The DNA from these modified vectors can be amplified in E. coli and then used to transform yeast. By transforming leu 2 asporogenous strains to prototrophy, it is then possible to screen transformed clones for the presence of DNA inserts that complement known mutations causing asporogeny.

Since the asporogenous strains characterised previously, 69.10C and XN129, did not carry leu2 markers, suitable leu 2-3 leu 2-112

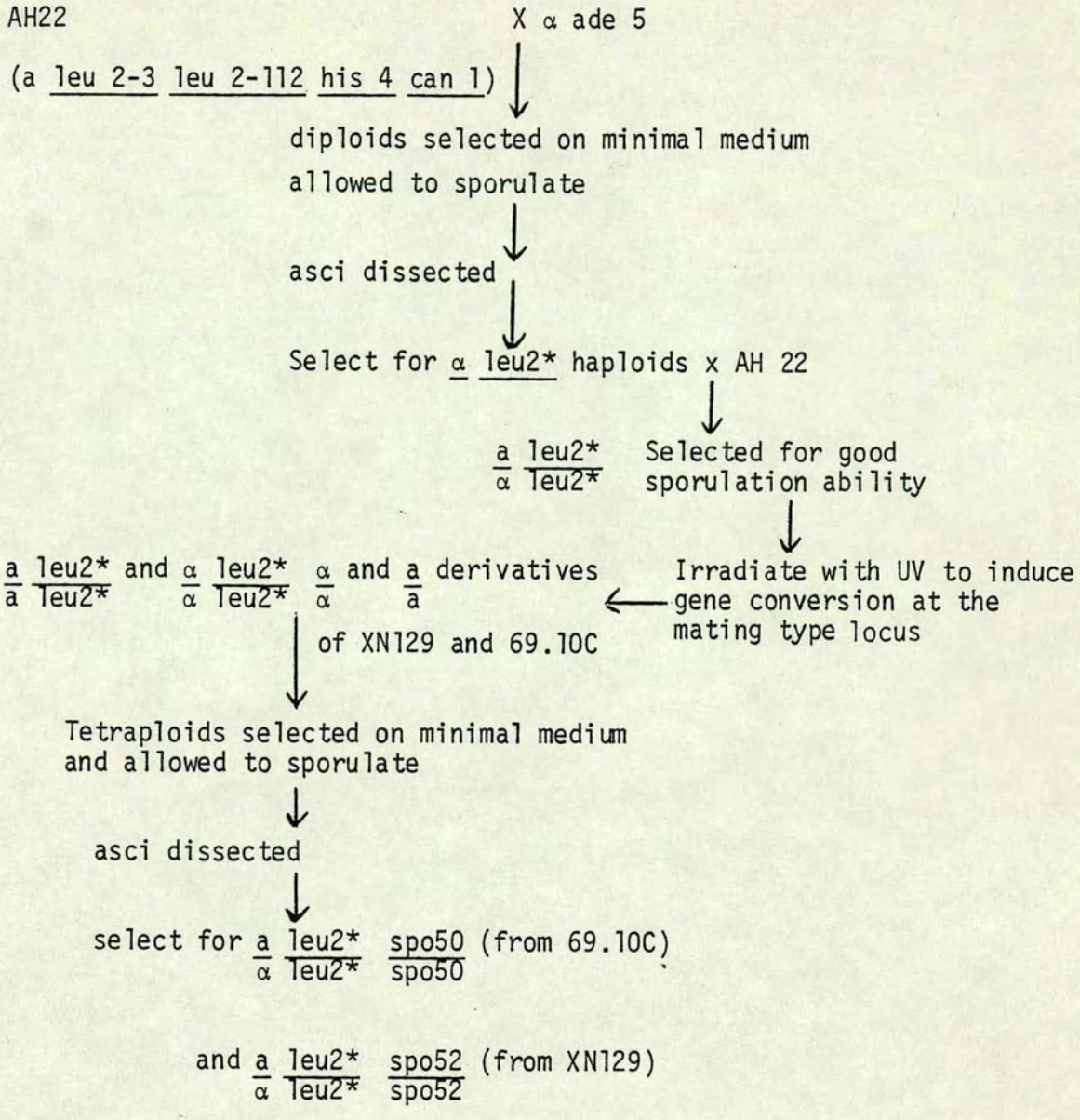


Figure 6.4 : Transfer of leu2* markers into asporogenous mutants 69.10C and XN129.

leu2* indicates the leu2-3 leu2-112 combination

spo strains had to be constructed and Figure 6.4 summarises the steps taken. Strain AH22 was crossed to αade5 to obtain diploids; sporulation and dissection allowed the isolation of αleu2* progeny which were crossed back to AH22 to give diploids homozygous for leu2*. Clones that sporulated well were selected and UV-irradiated to induce gene conversion at the mating-type locus. a/a and α/α derivatives were crossed to a/a and α/α strains derived from XN129 and 69.10C that had previously been isolated (Section III). The resultant tetraploids were sporulated and asci dissected; ascospores were screened for a/α diploids that required leucine and that were not able to sporulate. Reversion tests by short exposure to UV radiation on selected a/α leu2*/leu2* spo/spo diploids plated at high cell density on complete medium minus leucine (at least 12 plates for each diploid) showed that the leu2* marker in the diploids was stable.

Transformation of diploid leu2* strain to LEU$^+$ phenotype

Protoplasts were prepared from the derivatives of strains XN129 and 69.10C that were carrying leu2* markers. Purified plasmid DNA was mixed with the protoplasts and these were plated on appropriate selective media.

About 20% of the treated protoplasts plated on supplemented minimal medium were able to regenerate cell walls and formed colonies (similar to AH22). No transformation was observed however, when samples were plated on supplemented minimal medium lacking leucine. Transformation was further attempted using another hybrid plasmid YEp13 which also carries the LEU2 yeast gene and a DNA fragment containing

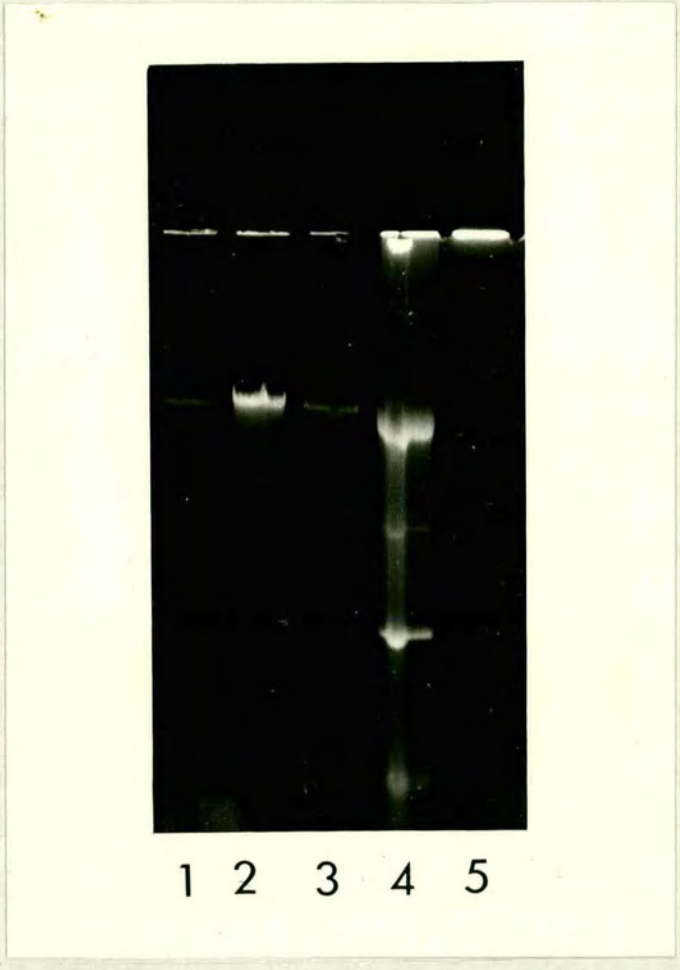


Figure 6.5 : DNA prepared from derivative of XN129 transformed to LEU^+ with YEp13, electrophoresed on 1% (w/v) vertical agarose gel and stained with ethidium bromide. The sources of DNA were:
(1) (2) & (3) transformed leu2* derivatives of XN129 (4) YEp13 prepared from transformed E. coli strain HB101 (5) The untransformed leu2* derivative of XN129.

an origin of replication from the yeast 2μ circular DNA (donated by Broach et al., 1979; Cold Spring Harbour Laboratory). YEp13 was propagated and prepared by similar techniques to those used for pJDB207. The 69.10C derivative was still not able to form colonies after the transformation procedure but XN129 was transformed at a low efficiency of 1 in 10^8 protoplasts. Repeated attempts consistently produced similar results. Figure 6.5 illustrates plasmid DNA prepared from the transformed derivative of XN129 after electrophoresis on 1% agarose slab gels after staining with ethidium bromide. The plasmid prepared from the transformed yeast appeared to be larger than YEp 13. Most likely, once taken up by the host, plasmid YEp13 underwent rearrangement, for example it may have recombined with the 2μ circular yeast DNA, retaining the LEU2 gene which was expressed and enabled growth on medium lacking leucine. Alteration of plasmids in yeast after their uptake by a host is not at all uncommon, and has been reported by other workers. (Beggs, 1978; Broach et al., 1979; Gerbaud et al., 1979).

Although the results were poor in terms of transformation frequency, they indicated that it was possible to transform diploid cells using hybrid plasmids, since the derivative of XN129 was able to take up YEp 13 as shown by complementation of its leucine requirement. Strain differences probably accounted for the poor results and the inability of derivatives of strain 69.10C to take up or harbour any of the plasmids. The transformation techniques clearly require further modification for them to be successfully employed with different strains of yeast. Other plasmids should be constructed and tested as potential vectors, since the efficiency of yeast protoplasts

to take up and to maintain different plasmids probably differs. Improvement in technique, the availability of a suitable vector, and construction of a vector-yeast DNA bank would enable the isolation of sporulation-specific genes from yeast. Strains such as the leu2* derivative of XN129 would make ideal asporogenous hosts for such transformation experiments since, as indicated earlier, the spo52 mutation to asporogeny had a very low reversion frequency and it would also be recognised by the sensitivity to UV radiation that it confers on homozygous diploids. This would facilitate the screening of potential transformants.

At this point no time was available to continue the isolation of the spo52 gene, however the above work has shown that the project is feasible and has provided a well-characterised yeast strain that can be used. This work is currently being extended in the laboratory.

GENERAL DISCUSSION

When the research for this thesis was begun, the morphological changes taking place during sporulation had been characterised in detail, a few biochemical events that could be considered to be specific to the process were known, and genetic analysis had indicated that roughly 50 genes were involved in functions indispensible to sporulation (Esposito et al, 1972). Attempts had already been made to find specific protein changes, as a first step to an understanding of the regulation of gene expression during spore formation. However, these, and subsequent experiments done by the method of pulse-labelling sporulating cells with L - [^{35}S]-methionine failed to show any changes in the 400 major polypeptides of sporulating a/α cells that did not also occur in non-sporulating diploids (a/a or α/α) or even in haploids exposed to similar conditions (Petersen, et al., 1979; Trew et al., 1979). Changes were seen to occur, but these were found in all types of cells regardless of whether or not meiosis or spore formation occurred. This work, therefore, posed something of a dilemma to those interested in the control of gene expression during this simple example of cell development in an eukaryote. There was apparently no sporulation-specific control exerted over the synthesis of the 400 most readily detectable polypeptides in the cell, or pulse-labelling was not giving a clear picture of the changes accompanying sporulation.

One possible explanation for the lack of success in pulse-labelling experiments emerged from the study of the heterogeneity of sporulating populations reported in Section I of the results. Normally sporulation is induced by transferring exponential cultures

adapted to respiration into potassium acetate medium. In this situation only cells in G1 phase were able to undergo sporulation immediately. Cells in other stages of the cycle had to progress to G1 before attaining the ability to initiate sporulation (Esposito & Esposito, 1974a). Thus sporulating populations tend to be heterogenous as shown by the fractions separated on Urografin and Percoll gradients. In some biochemical studies a certain level of heterogeneity may be tolerated. However, this may not be the case when examining sequential events as in attempting to show sporulation-specific polypeptide changes.

It has also been shown that the rise in pH of sporulation medium which accompanies the sporulation process has an inhibitory effect on the cell's ability to take up precursors (Mills, 1972). Hence pulse-labelling may not be a suitable technique to examine sporulation-specific changes. Even the usual practice of resuspending the culture into buffer at pH6 did not improve uptake. Most of the labelled precursors were taken up by cells which were not yet committed to meiosis or sporulation.

In order to avoid these problems, another approach to labelling proteins during sporulation was developed by Wright & Dawes (1979). This technique involved the continuous labelling of cellular proteins with $^{35}SO_4^{2-}$ during presporulation growth and then resuspending the cells in sulphur and label-free sporulation medium. In this way any modification to the vegetative cell proteins would have been detected and, since sporulating cells utilise internal pools of macromolecule precursors, newly synthesised

proteins would also have been labelled. This technique has enabled the detection of several sporulation-specific polypeptide changes (from amongst the 400 major polypeptides) during sporulation of Saccharomyces cerevisiae.

By employing a similar technique of labelling, this work has been extended as reported in this thesis. The results of the various experiments have been discussed previously in some detail at the end of each section. However, it is now possible to summarise more fully the nature and timing of the changes that occur during sporulation in Saccharomyces cerevisiae under the conditions that have been used here. This has been done in Table 3, which draws together the data from this thesis as well as some work done in conjunction with Dr. John Wright on phosphorylation. From these data the following general conclusions can be drawn:

1. There are about 21 changes (in 400 detectable polypeptides) that are unique to sporulation cells placed under sporulation conditions. There are other changes that occur during sporulation of a/α cells, but these also take place in non-sporulating (a/a and α/α) diploids or haploids placed under similar nutritional conditions. THis is to be expected since sporulation is induced by a change in nutritional conditions, occurring on transfer of growing cells to a starvation medium. These adaptive changes may however, still be (indirectly) important to sporulation, enabling cells to redirect their metabolism to support either sporulation or changes needed to survive starvation.

2. The polypeptide changes that were unique to sporulation occurred sequentially over a long time span; while most of them were detected as early events, some took place later when morphological changes were well underway. There is therefore some control exerted over protein changes in sporulating cells.

3. From a study of two mutants that are blocked early in sporulation, one possibly at initiation, the other just after the onset of DNA replication and meiotic recombination, it was shown that these early events could also be 'ordered' since only a few occurred in the earliest blocked, and in that blocked later the same changes had occurred, but another set had also appeared. The mutant studies have also indicated that the polypeptide changes follow a dependent sequence in which most later events are only begun if most earlier events have taken place, as has been clearly shown (for a greater number of mutants) in bacterial sporulation (Piggot & Coote, 1976). Clearly many more mutants need to be studied in this way before it is possible to identify groups of polypeptides that are co-regulated.

4. The polypeptide changes accompanying sporulation that have been described here could have been due to de novo synthesis, or to modifications of existing 'vegetative' cell polypeptides. Modifications could, however, depend on protein synthesis, since modifying enzymes may have to be synthesised. Clearly, if one wishes to ascribe some of the regulation of sporulation to the level of gene expression, it is necessary to distinguish between these possibilities. So far, the possible involvement of phosphorylation as a modification mechanism has been indicated, (Wright et al., 1981) since ten of the polypeptides that were

changing during sporulation could be labelled by [^{32}P]-orthophosphate, and included amongst these were three sporulation-specific ones (7, 20 and 41). Several other early changes may well have been due to modifications since they took place even in the presence of cycloheximide, the protein-synthesis inhibitor, as illustrated by polypeptide 20 (sporulation-specific) which was phosphorylated at the time of its appearance as well as its synthesis being resistant to cycloheximide. The appearance of polypeptide 20 probably involved modification by phosphorylation and this was brought about by enzyme(s) already present in the sporulating cells. On the other hand several other early changes were sensitive to cycloheximide, and these therefore were dependent on *de novo* protein synthesis. While such changes may still be due to modifications it seems likely that some at least are the result of the expression of genes that are uniquely read during sporulation.

Further work is necessary to resolve these points. For example, inhibitors of mitochondrial protein synthesis, such as chloramphenicol could be used to distinguish whether those polypeptides resistance to cycloheximide were due to modifications by existing enzymes or proteins synthesised in mitochondria. Two other approaches include; *in vitro* translation of RNA isolated from sporulating cells, to show unique transcripts and gene cloning. These are discussed later.

The sporulation-specific polypeptide changes observed in the a/α are, therefore, probably reflecting in part a developmental programme of gene expression which gradually unfolds during sporulation. There is definite control over the timing of the

Table 3: Summary of all available data on polypeptide changes during sporulation of Saccharomyces cerevisiae

Polypeptides are numbered according to the scheme given in Figure 2.7. Labelling of cells proteins with $^{35}SO_4^{2-}$ and analysis by two dimensional gel electrophonesis were described fully in the methods section. Technique of labelling with ^{32}P-orthorphosphate was described in Wright et al. 1981. Determination of resistance to cycloheximide was illustrated in **Table** 4.4.

t - Time indicated was hours after resuspension into sporulation medium.

* - Results for a/α wild type diploid. Data from Wright, J.F. (1981).

\+ - Polypeptide phosphorylated during resuspension into sporulation medium.

▽ - Results obtained from 69.10C and XN129 only.

\- - Polypeptide not present.

R - Polypeptide resistance to cycloheximide, 1 and 2 refer to the appearance in mutants 69.10C and XN129 respectively.

No entry - No changes observed.

Polypeptides / Sporulation specific NEW	[t] Time of 1st appearance			[*] Phosphorylation	[▽] Response to cycloheximide
	JW1	69.10C	XN129		
1	24				
2	24				
7	2			+	
11	4		4		
16	4	4	4		
17	2		2		
20	2	2	2	+	$R^{(1\&2)}$
23	4	4	4		
24	6	6			
26	2	2	2		$R^{(1\&2)}$
27	2		2		$R(2)$
35	2		2		
38	4				
39	6				
Concentration increase					
4	2				
10	4		4		
13	2				
14	2				
28	8	-	-		
37	2	-			
45	4	-	-		
Common Appearances NEW					
12	4	4	4		
29	2	2	2		
33	6	6	6		
40	2	6	6		
43	2	2	2		
44	2	2	2		
Concentration increase					
3	4	4	4		
5	2	2	2	+	
18	2	2	2		
19	2	2	2		
21	6		6		
22	6		6		
30	6		6	+	
31	4	4	4		
32	4	4	4		
34	6		6		
36	6		6		

Other Alterations					
6	24				
8	6	6	-		
9	6	6			
15	6		6		
25	24				
41	4			+	
42	8	-	8		

changes, however, it is not known at what level (e.g. transcription, translation, or post translational) any alteration during sporulation is regulated. Most likely, control is exerted at each of these levels in particular cases.

Levels of Regulation

The initiation of sporulation and the derepression of catabolite-repressible enzyme synthesis are induced by similar conditions. There is therefore a tendency to think of derepression of sporulation as involving similar contol mechanisms to those seen in bacteria in which regulatory proteins direct the transcription of certain catabolite-sensitive operons in response to glucose or nitrogen deprivation. However, it must be noted that the initiation of sporulation normally required the deprivation of both fermentable carbon and nitrogen sources. Successful induction is confined to a specific part of the cell cycle and, most significantly, only cells that are heterozygous at the mating-type locus are able to sporulate. This emphasises the complexity of the control mechanisms involved and indicates that it may be rewarding to analyse transcriptional control of the polypeptide changes.

One of the most attractive hypothesis to explain changes in specificity of transcription during sporulation suggests that structural changes occur in RNA polymerase II which alters its specificity. Like other eukaryotic organisms, Saccharomyces cerevisiae has multiple forms of DNA-dependent RNA-polymerase and these can be separated by DEAE-Sephadex ion exchange chromatography

and distinguished by their sensitivity to α-amanitin (Sebastian et al., 1973). Magee (1974) reported that changes occurred during sporulation in the pattern of RNA polymerase activity eluted from a DEAE-sephadex column. A new species of the polymerase II enzyme that is responsible for mRNA and sensitive to α-amanitin appeared after 6h sporulation. Further experiments done by Klar et al. (1976) were unable to confirm this result. However, the authors did not rule out that subtle structural changes in the enzyme may have occurred that did not lead to changes in its chromatographic properties.

Control at the transcription level is further suggested by the findings of Curiale & Mills (1975) who showed that a significant amount of new mRNA was synthesised during the early part of sporulation. Recently, Mills, (1980) has further confirmed this by showing that there are some polyadenylated RNA species present in a/α sporulating cells that are absent in α/α cells exposed to similar conditions. These findings were consistent with the pattern of polypeptide changes observed on the 2D PAGE analysis. Many of the new polypeptide appearances and alterations occurred during the early part of sporulation.

The synthesis during sporulation of mRNA species unique to sporulating cells has also been shown by in vitro protein synthesis studies using as template polyadenylated RNA prepared at intervals during sporulation. New polypeptide bands that were absent in the a/a strain were observed in an a/α sporulating diploid, after translation using the rabbit

reticulocyte lysate system and separation of the products on one-dimensional gel electrophonesis (G.R. Calvert & E.M. Weir-Thompson, personal communication).

So far, the only indication that there may be regulation at the translational level during sporulation is the change in the amount of different isoacceptor species of tRNA reported by Sogin et al (1972). Mills (1974) isolated polyribosomes during sporulation and exponential growth. The results however did not show any differences that would indicate control at this level. Despite the lack of evidence, it does not necessarily imply that control at the translation level is not involved in sporulation. Translation in eukaryotes has been well studied and there are many possible points at which control can occur.

Post-translational controls involving protein modifications have been shown in this thesis. Modification at this stage could include selective proteolysis, methylation, adenylation and as illustrated by Wright et al., (1981) modification by phosphorylation. All these modifications may still, however, involve de novo synthesized proteins, such as enzymes that are repressed during vegetative growth.

Suggestions for future research

The work done for this thesis has highlighted several areas in the study of regulation of yeast sporulation in which research should be continued to give a better understanding of the process.

As discussed at the end of section IV of the results, the data on the nature of polypeptide changes should be extended by using other sporulation mutants and selective inhibitors such as 8 hydroxyquinoline to inhibit transcription and chloramphenicol to inhibit mitochondrial protein synthesis. Examining proteins synthesised in mitochondria could indicate their relevance to sporulation, although it has been shown that Saccharomyces cerevisiae can sporulate in the possible absence of mitochondrial protein synthesis.

Another area which would give further understanding of the regulation mechanisms involved in gene expression during sporulation, is to employ the recently available techniques of transformation and gene cloning in yeast. The preliminary steps towards the cloning of sporulation genes had already been attempted in Section VI. Successful isolation and characteristion of sporulation-specific genes would contribute a lot to the little information currently available on the regulation of sporulation at the genetic level.

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List of publications

1. Separation on Urografin Gradients of Subpopulations from Sporulating Saccharomyces cerevisiae Cultures. Ian W. Dawes, John, F. Wright, Francoise Vezinhet and Noni Ajam (1980). Journal of General Microbiology 119, 165-171.

2. Timing of Specific Protein Changes during Yeast Sporulation. Noni Ajam, John F. Wright and Ian W. Dawes. In 'The Abstract of VIth International Fermentation Symposium and Vth International Symposium on Yeasts (1980). p.229.

3. Sporulation Specific polypeptide changes in Asporogenous Mutants of Saccharomyces cerevisiae. N. Ajam and I.W. Dawes (1981). 'Society for General Microbiology Quarterly, 8, p. 249.

4. The Nature and Timing of some sporulation-specific protein changes in Saccharomyces cerevisiae. Wright, J.F., Ajam, N. & Dawes, I.W. (1981). Molecular and Cellular Biology 1, 910-918.

Separation on Urografin Gradients of Subpopulations from Sporulating *Saccharomyces cerevisiae* Cultures

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Sporulating yeast populations were separated into a number of fractions by centrifugation through linear density gradients of Urografin. At least eight distinct fractions could be obtained from cultures that were just beginning to produce asci visible by phase contrast microscopy. Cells in these fractions were found to differ in the stage they had reached in meiosis and sporulation; those more advanced in sporulation banded at a lower density. Using this gradient centrifugation method it was shown that cultures induced to sporulate by the usual technique were markedly heterogeneous with respect to the stage in meiosis and sporulation reached by the cells, and that most of the labelled amino acids incorporated into protein by sporulating cultures appeared in those cells not yet committed to either recombination or meiosis.

INTRODUCTION

Saccharomyces cerevisiae provides a system in which meiosis and the subsequent developmental system of ascospore formation are amenable to biochemical and genetic analysis. For most purposes the processes can be induced in an adequate proportion of cells in a culture (usually between 70 and 80%) with a reasonable degree of synchrony by using the resuspension techniques of either Roth & Halvorson (1969) or Fast (1973). There are, however, situations in which asynchrony and the presence of non-sporulating cells in such cultures can distort the results obtained; this is particularly so when sequential changes are under study and pulse-labelling techniques are in use. A procedure has been described that is based on sporulation of cultures synchronized with respect to cell division (Sando *et al.*, 1973), but this is still subject to the problem of incomplete induction of sporulation in the population.

Here we describe a technique that resolves sporulating populations into fractions conaining cells at different stages of the processes of meiosis and sporulation. Using this method we have been able to illustrate the heterogeneity of cultures induced to sporulate according to the procedure of Fast (1973) and to show marked differences between cells in the various fractions with respect to the incorporation of labelled amino acids. These results highlight the problems associated with pulse-labelling studies since most of the label incorporated into a 16 h sporulation culture containing 1% asci was found in cells which had not even become committed to meiosis.

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METHODS

Strain. Saccharomyces cerevisiae strain $60 \times 61 \left(\frac{a\ HO\alpha\ HM\ ade\ 2\text{-}40\ his\ 4\text{-}239\ ARG\ 4\text{-}1\ MET\ 13\ can\ 1}{\alpha\ HO\alpha\ HM\ ade\ 2\text{-}119\ his\ 4\text{-}166\ arg\ 4\text{-}1\ met\ 13\ CAN\ 1} \frac{CYH\ 2}{cyh\ 2}\right)$ was constructed from a variety of strains including some supplied by Dr F. Zimmerman, Darmstadt, F.R.G., and the Yeast Genetics Stock Culture Collection, Berkeley, U.S.A. The *ade 2–40* and *ade 2–119* alleles complement and the parent diploid is adenine-independent with white colonies. However, once meiosis and segregation of the nuclei has occurred, the haploid progeny require adenine and give colonies that are either red (*ade 2–40*) or pink (*ade 2–119*, a leaky mutation). This means that cells committed to completing meiosis can be scored directly after plating on any medium. Cells committed to recombination can be scored by testing for loss of the histidine requirement due to recombination between the non-complementing alleles of the *his 4* gene complex. *HOα* and *HM* are genes concerned with homothallism. The genes *can 1* and *cyh 2* confer recessive resistances to L-canavanine sulphate (80 μg ml^{-1}) and to cycloheximide (5 μg ml^{-1}), respectively; these genes are both present in the heterozygous state and the appearances of resistant organisms can also be used as marker events during sporulation. The strain was maintained on YEPD agar [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose, 2 % (w/v) agar] at 0 °C, and was cloned before use.

Sporulation. Cultures were grown to a turbidity of 1 (600 nm) on YPA medium^{r} [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) potassium acetate] and were induced to sporulate, according to the method of Fast (1973), by resuspension in 2 % (w/v) potassium acetate, pH 5·5, supplemented with the appropriate auxotrophic requirements.

Incidence of asci and buds. The percentage of asci in each fraction was determined by direct counting using phase contrast microscopy. There is in this method an element of subjective judgement since the developing ascospores can be discerned before they appear as distinct and separate entities. This was most noticeable in scoring fractions since different fractions contained different stages in the development of the ascus. Buds were counted as separate cells, and, where possible, sporulating cultures with a low proportion of budded cells were used.

Uptake and incorporation of labelled amino acids. Separate uptake and incorporation studies were done using either L-[^{14}C]phenylalanine or L-[^{14}C]arginine. The sporulating culture (3 ml) was either labelled directly by adding the labelled amino acid as described below, or it was first centrifuged at 3000 *g* for 2 min and the cell pellet was resuspended in the same volume of either 2 % (w/v) potassium acetate adjusted to pH 6, or 2 % (w/v) potassium acetate buffered to pH 6 with 0·2 M-morpholinopropanesulphonic acid (MOPS). The resuspension medium was warmed to 30 °C before use, and contained L-[^{14}C]arginine at 5 μCi ml^{-1} (185 kBq ml^{-1}) and 5 μg ml^{-1}, or L-[^{14}C]phenylalanine at 5 μCi ml^{-1} (185 kBq ml^{-1}) and 5 μg ml^{-1}. After a pulse of 10 min, a sample (1 ml) was gently layered over the Urografin (Schering Chemicals Ltd, Burgess Hill, West Sussex) gradient and centrifuged immediately (see below). Under these conditions uptake refers to the amount of ^{14}C retained by the cells after centrifugation through the Urografin gradient. Samples (10 μl) from each fraction obtained from the gradient were transferred directly into scintillant (1 ml Insta-Gel, Packard Instrument Co.) and counted.

Incorporation into trichloroacetic acid (TCA)-precipitable material was measured by taking 0·1 ml samples from each fraction into 10 % (w/v) TCA at 0 °C and, after 30 min, filtering on nitrocellulose filters (0·45 μm pore size). The filters were counted in 3 ml of 0·4 % (w/v) 2,5-diphenyloxazole in toluene.

Density-gradient centrifugation. Linear Urografin density gradients were prepared in 16·5 ml capacity polycarbonate centrifuge tubes (MSE code 0178) over the density range specified, usually within the limits of 1·13 to 1·22 g cm^{-3}. Best separations were achieved using a density range of about 0·06 g cm^{-3}, and since these were quite shallow gradients, experiments were often carried out using several gradients with different ranges and that yielding the best separation was used for analysis. Samples (1 ml) of sporulating cultures (containing about 10^{8} cells) were gently layered on the gradients and the tubes were centrifuged at 4500 *g* (max.) for 10 min in an MSE Super-Minor centrifuge with swing-out rotor. Fractions (0·3 ml) were obtained by piercing the tube and collecting from the bottom of the gradient.

Density estimation. The density of each fraction was determined by measuring its refractive index and comparing this with a standard curve prepared from Urografin solutions of known density.

Cell concentration and viability. Cell concentrations were estimated in terms of the turbidity of samples at 600 nm. Samples were diluted 10- or 20-fold to reduce the effect of refractive index changes due to the presence of Urografin. Cell viability was estimated by plating appropriate dilutions of the fractions on YEPD plates. After incubation of these plates for at least 3 d at 30 °C it was possible to score for the red/pink character displayed by the *ade 2* auxotrophs, as well as to estimate the total viable count.

Sectored colonies were presumed to have arisen either by recombination or segregation within an ascus,

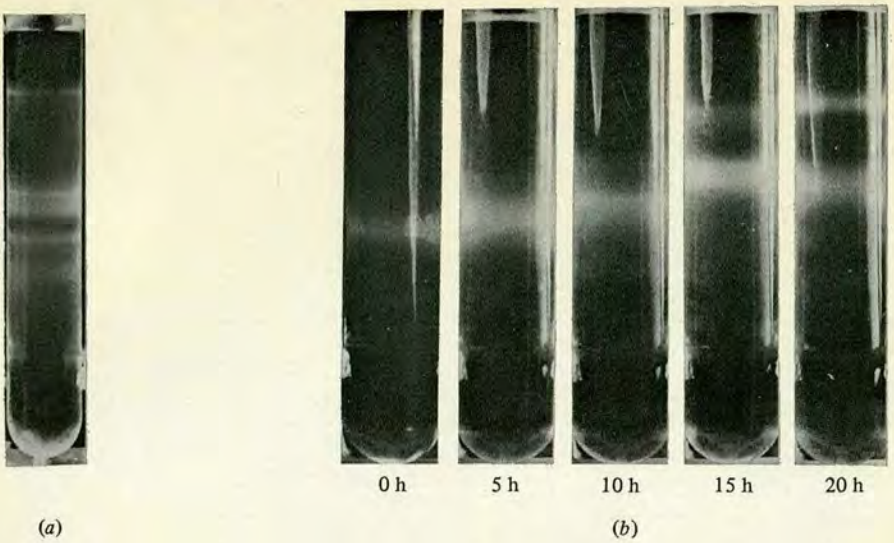

Fig. 1. Separation of sporulating cultures into subpopulations. (*a*) The pattern of cell banding observed on centrifugation of a 20 h sporulation culture through a linear gradient of Urografin (centrifugation time, 15 min; density range, 1·13 to 1·20 g cm^{-3}). (*b*) The effect of sporulation time: samples taken at the times indicated after resuspension in sporulation medium were separated on Urografin gradients (centrifugation for 15 min; density range, 1·13 to 1·22 g cm^{-3}).

and were scored as single viable units. Urografin did not appear to affect the viability of cells at any stage of sporulation.

Commitment to recombination. Fractions were diluted appropriately, plated on defined complete medium plates (Dawes *et al.*, 1977) lacking histidine as supplement and the plates were incubated at 30 °C for at least 5 d. Colonies were counted and the ratio of histidine-independent to total viable units was taken as an indication of the extent of commitment to intragenic recombination (Esposito & Esposito, 1974).

RESULTS

Separation of sporulating cultures on Urografin gradients

Initial experiments using linear Urografin gradients covering various density ranges indicated that sporulating yeast populations could be resolved into a number of bands by centrifugation, whereas under the same conditions vegetative populations were not. Best results (for example Fig. 1 *a*) were obtained by layering 1 ml of culture over a very shallow gradient (density 1·15 to 1·20 g cm^{-3}) in long tubes (97 mm, 16·5 ml capacity) and centrifuging at low speed (4500 *g*) for 10 to 15 min. These centrifugation times were not long enough for cells to attain their eventual equilibrium position. The changes in cell banding pattern with centrifugation time were found to be complex (some cells even moved to a high density position and then to one of lower density), and the exact procedure for any given yeast strain had to be determined on each occasion.

Resolution of cultures at different times after induction of sporulation

Figure 1 (*b*) shows the changes in the pattern of cell banding when cultures were separated (centrifugation for 15 min) at intervals of 5 h throughout sporulation. Several points were evident from these results. First, the presence of discrete bands in many of the samples indicates that there are distinct physical changes (presumably reflecting different physiological states) undergone by cells placed under sporulation conditions, and that the transition of cells from one state to another is fairly rapid, otherwise the cells would have been distributed much more broadly. Secondly, significant changes occur during sporulation

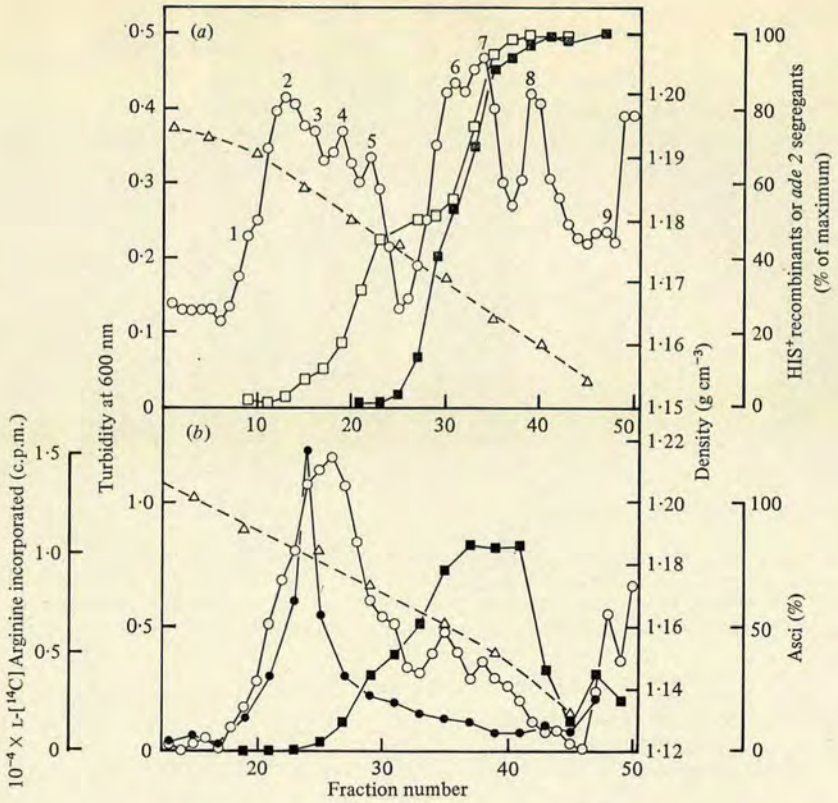

Fig. 2. Heterogeneity of sporulating cultures. (*a*) A 16·5 h sporulation culture was separated on a linear gradient (1·15 to 1·195 g cm^{-3}). The tube was pierced, fractions were collected from below and the following were estimated: turbidity at 600 nm (○); commitment to recombination at the *his 4* locus (□); commitment to meiosis (■); density of Urografin (△). Results are shown as a percentage of the maximum values for recombination ($6{\cdot}1 \times 10^3$ HIS$^+$ ml^{-1}) and meiosis (35% red-sectored colonies). Peaks are numbered 1 to 9. (*b*) Incorporation of L-[^{14}C]arginine by subpopulations in a 21 h sporulation culture. After pulse-labelling with arginine for 10 min the culture was separated on a linear gradient (1·13 to 1·20 g cm^{-3}). Fractions were collected and assayed for L-arginine incorporated into TCA-precipitable material (●). The following were also estimated: turbidity at 600 nm (○); percentage of asci (■); density of Urografin (△).

leading to an increased complexity of the banding pattern obtained at later sporulation times. As sporulation proceeded, fractions of apparently lower density appeared; most obvious was the uppermost band seen in Fig. 1(*b*). Thirdly, there is a marked increase in the heterogeneity of sporulating cultures with time after resuspension under conditions supporting meiosis and sporulation. The different fractions were therefore isolated and analysed to see if they contained cells at different stages of meiosis and sporulation.

Heterogeneity of sporulating cultures

The different bands obtained on Urografin gradients did contain cells in different stages of meiosis and sporulation. This was shown by separating sporulating cultures, collecting fractions and analysing them for events characteristic of sporulation. These included: commitment to recombination (by scoring for intragenic recombination at the *his 4* locus); commitment to meiosis (by segregation of the *ade 2* gene); and appearance of recognizable asci. In addition, cell turbidity and density were estimated.

Results are plotted in Fig. 2(*a*) for a 16·5 h sporulation culture; the separation achieved

in this experiment was one of the best obtained using a very shallow gradient. In the strain used, tetrads normally began to appear between 15 and 16 h after resuspension in sporulation medium, and these reached a maximum of about 70% of the population at 24 to 30 h. Most (80%) of the asci were four-spored under the conditions used. From Fig. 2(*a*) it can be seen that cells in the lower, denser part of the gradient were uncommitted to either recombination or meiosis, and that only those in the upper bands (numbered 6 to 9) showed any signs of segregation of the *ade 2* markers. Histidine-independent recombinants were detected in band 5 as well as in bands 6 to 9, but the maximum frequency of recombinants was not seen until band 7. Most cells in bands 1 to 5 were unbudded, and no asci were seen on microscopic examination. In band 6 no mature asci were found, but the population contained a high proportion of cells in which the first signs of ascus formation were just evident. Band 7 contained approximately 10% mature asci; the remaining cells were immature asci similar to those seen in band 6. Band 8 contained more than 60% asci and cells found above this were almost all mature asci.

It is evident from these results that 16·5 h after resuspension in sporulation medium about half of the population was not yet committed to meiotic recombination (at the *his 4* locus), although some had completed recombination, meiosis and formation of mature ascospores. This indicated that sporulating cultures contained cells that had progressed through meiosis and sporulation to different extents. Clearly, any measurement made on an unresolved sample taken at a particular time from such a population could give a misleading impression of events taking place at a particular stage of sporulation. This was most obvious in studies of the uptake and incorporation of amino acids by sporulating populations.

Uptake and incorporation of labelled amino acids

Pulse-labelling techniques have considerable use in tracing the fate of metabolites, or in studying the synthesis of particular molecules, but the results of such studies need to be viewed with caution when using heterogeneous populations. In the light of the above results, and the fact that sporulating cultures incorporate precursors of macromolecules at a much lower rate than vegetatively growing ones (Esposito *et al.*, 1970; Mills, 1972; Magee & Hopper, 1974), the uptake and incorporation of two amino acids, L-phenylalanine and L-arginine, by the different subpopulations in sporulating cultures was examined. In these experiments two methods were used to label the cultures. The first involved the direct addition of the labelled amino acid to a sporulating culture, and after 10 min incubation it was centrifuged through an appropriate Urografin gradient. The second method was that of Mills (1972) in which cells from sporulating cultures were harvested by rapid centrifugation and resuspended in potassium acetate (0·2 M) buffered to pH 6 prior to the addition of the labelled amino acid.

The results from both methods were qualitatively similar; those presented here were obtained using potassium acetate at pH 6. Figure 2(*b*) shows the extent to which the different cell types took up and incorporated L-[^{14}C]arginine when a 21 h culture containing 20% asci was pulse-labelled prior to separation on a Urografin gradient. In this experiment both the total radioactivity taken up and that precipitable by cold 10% (w/v) TCA were estimated. The distribution of total activity and of TCA-insoluble activity was similar except at the top of the gradient where unincorporated label remained during centrifugation. A very marked difference was seen between different cell fractions in the extent of uptake and incorporation of label (relative to the concentration of cells in each fraction), and it was clear that most of the label was taken up by those cells that were not yet committed to meiosis.

This experiment was repeated using a different amino acid, L-phenylalanine, to see whether the effect was limited to arginine, and also using an earlier culture (16 h) under improved separation conditions. Again, most of the labelled precursor taken up during a 10 min pulse was found in those cells that had not yet begun to undergo meiosis or sporulation, or that were at any early stage in meiosis.

DISCUSSION

The separation of sporulating cultures on Urografin gradients enables resolution of a single culture into fractions containing cells at certain physiological stages of meiosis or sporulation. For example, it was possible to obtain a fraction of cells that were committed to meiotic recombination but not yet committed to meiotic segregation. With care, and using shallow gradients, up to seven or eight fractions can be obtained. The correlation of these physiological stages with the morphology of ascospore formation is currently under study, but it is clear from the results presented here that as cells progress further into sporulation they band at positions of lower density in Urografin gradients. Moreover, they must undergo the transition from one state to the next in a fairly rapid manner. Since the separation method does not depend on density alone, the physiological basis for these changes is not obvious. The changes in lipid synthesis (Henry & Halvorson, 1973) that accompany sporulation would lead to a decrease in cell density and could contribute to the effect; other processes which may be important include the breakdown of glycogen (Kane & Roth, 1974) and the synthesis of the spore coat structures which may alter the density of the ascus and the permeability of parts of it. One of the main shifts in banding position of the cells seems to begin when the outline of developing ascospores can just be discerned by phase contrast microscopy, corresponding to the change from band 5 to band 6 in Fig. 2(*a*).

Several problems were encountered using this method. First, rather large amounts of Urografin were needed to separate relatively small samples, and unless the Urografin were to be recovered the technique could prove expensive if large amounts of material were needed for subsequent analysis. Other materials, including Ficoll and colloidal silica, were tried with no advantage over Urografin being apparent. Different strains of *Saccharomyces cerevisiae* also required preliminary characterization to establish the useful density range for the gradients. A problem that arose on several occasions, and for which no clear explanation has been found, was the flocculation or aggregation of cells in the Urografin. A number of variables were tested, including the use of various salts, buffers of different pH, and lower sample loadings. None of these affected the aggregation markedly, although lowering the sample loading helped a little. In practice, the problem was remedied by recloning the yeast strain used. A note should also be made about the recovery of the bands from the centrifuge tubes after centrifugation. For the results presented here, fractions were obtained by pumping off the gradient from below via a peristaltic pump. It has recently been found that this leads to considerable broadening of the bands and better results can be achieved by avoiding pumps on the outlet side of the tube.

Despite the limitations, the technique has considerable potential for a number of biochemical studies of meiosis and sporulation in yeast since it enables resolution of cells at different physiological stages from a single culture.

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Sporulation and Conjugation

Y-3.3.1(P) *

Timing of Specific Protein Changes during Yeast Sporulation.
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Changes in polypeptides that are specific to sporulating yeast have been detailed. These may have been due to either modifications of existing protein, or to de novo protein synthesis (Wright & Dawes, 1979; FEBS Lett., 104, 103-180). These changes may account for up to 5% of the polypeptides detectable by two-dimensional gel electrophoresis.

Yeast cultures that had been prelabelled during vegetative growth with $^{35}SO_4^{2-}$ were analysed by two-dimensional gel electrophoresis at different times after resuspension in sulphur-free sporulation medium. The specific changes followed a time sequence, some occurred very early prior to the commitment of the cells to recombination or meiosis, while others took place much later, when asci were apparent in the cultures.

The establishment of this time sequence has enabled the characterisation of mutants affected at different stages of sporulation. The results should contribute to an understanding of the regulation of sequential changes, including modifications to existing proteins, during morphogenesis in a microbial eukaryote.

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G12 *Sporulation-Specific Polypeptide Changes in Asporogenous Mutants of Saccharomyces cerevisiae*. N. Ajam and I.W. Dawes (Department of Microbiology, University of Edinburgh, West Mains Road, Edinburgh EH9 3JG)

During meiosis and spore formation in *Saccharomyces cerevisiae* changes occurred in the cellular polypeptides of the a/α diploids but not in the isogenic non-sporulating a/a diploids. These were observed by prelabelling the growing cells with $^{35}SO_4^{2-}$ and resuspending them in unlabelled sporulation medium. Two-dimensional gel electrophoresis of the extracted protein detected about 400 polypeptides, 11% of which altered during sporulation (1). These changes were mainly due to the appearance of new polypeptides or to marked increases in the concentrations of a few vegetative polypeptides. They may be the products of new genes translation or modifications of existing polypeptides present in the growing cells. The changes occurred at characteristic times during sporulation. While the majority took place within the first 6 h after resuspension in sporulation medium, there were several changes characterizing the later stages of sporulation.

Polypeptides from the diploid sporulation-deficient mutants homozygous for either *spo 50* (blocked at initiation) or *spo 52* (blocked just after the onset of DNA synthesis) were analysed by a similar technique. Both showed a number of the changes that were observed in the a/α sporulating diploids. Four new polypeptides appeared in *spo 50* and eight in *spo 52*. All of them were early changes, occurring within the first 4 h after resuspension in sporulation medium. Most of them, however, did not show significant increases in concentrations as in the a/α diploids. This was also true for the vegetative polypeptides that increased in concentration during sporulation. In both mutants, they remained unchanged as in the non-sporulating a/a diploids.

1. Wright, J.F. and Dawes, I.W. (1979) *FEBS Letters* 104, 183.